

MECHANISMS OF MICROVASCULAR INFLAMMATION INDUCED BY ALVEOLAR HYPOXIA

BY

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INDUCED BY ALVEOLAR HYPOXIA**

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ABSTRACT

Alveolar hypoxia is observed in a number of clinical settings, and is frequently associated with systemic effects, many of which present an inflammatory component. Reduction of alveolar PO₂ in rats induces a rapid and widespread inflammatory response in mesentery, skeletal muscle and brain, characterized by increased microvascular levels of reactive oxygen species (ROS), increased leukocyte-endothelial adhesive interaction, extravasation of albumin and perivascular mast cell degranulation. There is substantial evidence that the systemic inflammation elicited by alveolar hypoxia is not triggered by the reduction of peripheral tissue PO₂, but rather by a mediator(s) released from alveolar macrophages and transported by the circulation. The mediator activates local tissue mast cells which release inflammatory agents and activate the renin-angiotensin system (RAS) to initiate the systemic inflammation.

The major objective of this study was to investigate the links between alveolar hypoxia, alveolar macrophages, resident tissue macrophages and mast cells to understand the mechanisms underlying the systemic inflammation.

Our results showed that topical application of supernatant of hypoxic alveolar macrophages, but not of hypoxic peritoneal macrophages produced inflammation in the normoxic mesentery. Hypoxia induced a respiratory burst in alveolar, but not peritoneal macrophages. Cultured peritoneal mast cells did not degranulate with hypoxia. Immersion of mast cells in supernatant of hypoxic alveolar macrophages, but not in supernatant of hypoxic peritoneal macrophages, induced mast cell degranulation. These data suggest that alveolar macrophage-

borne mediator activates mast cells and triggers the systemic inflammation induced by hypoxia, in which reduced systemic PO₂ and activation of tissue macrophage do not play a role.

Hypoxia induced release of monocyte chemoattractant protein-1 (MCP-1/CCL-2), a mast cell secretagogue, from alveolar macrophages, but not peritoneal macrophages or mast cells. Further studies showed that alveolar macrophage-borne MCP-1 played a central role in the inflammation: 1) Alveolar hypoxia produced a rapid increase in plasma MCP-1 concentration of conscious intact rats, but not of alveolar macrophage-depleted rats. 2) Degranulation occurred when mast cells were immersed in the plasma of hypoxic intact rats, but not of alveolar macrophage-depleted rats. 3) MCP-1 added to normoxic rat plasma and supernatant of normoxic alveolar macrophages produced concentration-dependent degranulation of immersed mast cells. 4) MCP-1 applied to the mesentery of normoxic intact rats replicated the inflammation of alveolar hypoxia. 5) The CCR2b receptor antagonist RS-102895 prevented the mesenteric inflammation of alveolar hypoxia in intact rats. Additional data suggested that a co-factor constitutively generated in alveolar macrophages and presented in normoxic body fluids is necessary for MCP-1 to activate mast cells at biologically relevant concentrations.

As previously seen in cremaster, the RAS is involved in the mesenteric inflammation of hypoxia. Demonstration of similar inflammatory pathways in both cremaster and mesentery provides further support to the idea of a circulating mediator initiating the inflammation of hypoxia. The previous findings in the

cremaster were expanded in several ways: an involvement of NADPH oxidase as an RAS effector was shown. In addition, it was demonstrated that renin is expressed in rat peritoneal mast cells, and that renin from MCP-1/CCL2-activated mast cells contributes to activation of tissue-specific RAS in inflammation induced by alveolar hypoxia.

In summary, the present studies provide substantial evidence in support of the idea that the systemic inflammatory response to alveolar hypoxia is initiated by an alveolar macrophage-borne MCP-1, which, in turn, activate local RAS via mast cell-derived renin and initiates the cascade of inflammation.

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CHAPTER 1

Introduction

Reduced alveolar PO_2 is observed in a number of clinical settings, and is frequently associated with systemic effects, many of which present an inflammatory component. Reduction of inspired PO_2 alveolar macrophage-induced systemic inflammation has been documented in humans and in animal experiments. The major objective of this project was to characterize a novel phenomenon, namely the systemic inflammation initiated by alveolar macrophages activated by a reduction of alveolar PO_2 . Investigation of the links between alveolar macrophages, alveolar hypoxia, and systemic inflammation provided insights into the pathogenesis of the systemic effects of conditions associated with alveolar hypoxia.

1.1 Alveolar hypoxia and systemic effects

Hypoxia, as a result of impairments in one or more of the linked steps of the O_2 transport system, is a very serious and common feature of many diseases of cardiovascular and respiratory origin. Frequently, clinical conditions as well as experimental interventions associated with alveolar hypoxia are accompanied by systemic effects. These systemic effects include cachexia and muscle wasting (Agusti and Soriano, 2008; Wust and Degens, 2007) and the cardiovascular abnormalities (Eickhoff et al.) of chronic obstructive pulmonary disease (COPD),

the suboptimal erythropoiesis of pulmonary fibrosis (Tsantes et al.) and the cardiovascular complications of sleep apnea (Jelic et al., 2008; Morgan, 2007). In acute conditions, systemic responses with a possible inflammatory link include the multiple organ failure secondary to atelectasis (Kisala et al.), acute lung injury (Puneet et al., 2005; St John et al., 1993), pulmonary contusion (Perl et al.) and the systemic inflammation of pneumonia (Fernandez-Serrano et al.). Alveolar hypoxia occurs also in healthy individual exposed to altitude. Rapid ascent to high altitude is increasingly frequent and may result in acute mountain sickness or high altitude cerebral edema (Basnyat and Murdoch). Although the role of inflammation in these cases remains unclear yet, the presence of elevated levels of circulating and tissue inflammatory markers (Beidleman et al., 2006; Hartmann et al., 2000; Klausen et al., 1997), and the effectiveness of dexamethasone, an anti-inflammatory steroid, in the treatment of acute mountain sickness (Basnyat and Murdoch, 2003; Michiels et al., 2000), suggest the contribution of an inflammatory component.

Rats breathing 10% O₂ show a rapid inflammatory response in mesentery, skeletal muscle and pial microcirculations within minutes of the onset of hypoxia. This response is characterized by increased levels of reactive oxygen species (ROS) (Wood et al., 1999a), mast cell degranulation (Steiner et al., 2003), leukocyte-endothelial adhesive interactions (Dix et al., 2003; Shah et al., 2003; Steiner et al., 2003; Wood et al., 1999b), and extravasation of albumin (Wood et al., 2000). Increased levels of ROS-dependent fluorescence occur within minutes

of the onset of hypoxia, and are observed in perivascular mast cells, the endothelial layer of postcapillary venules and the sites of leukocyte-endothelial adherence (Wood et al., 2000; Wood et al., 1999b). The magnitudes of the ROS-dependent fluorescence intensity and the leukocyte-endothelial adhesive interactions are inversely related to the PO_2 value (Steiner et al., 2002), and were significantly attenuated by the antioxidants SOD/catalase and lipoic acid (Wood et al., 1999a, 2000). Increasing microvascular NO levels by administration of a NO donor, spermine NONOate (sNO) or of the NO precursor L-arginine blocked the increases in ROS and in leukocyte-endothelial adhesive interactions induced by hypoxia (Steiner et al., 2002). This suggests that hypoxia is associated with a decrease in NO as well as an increase in ROS levels. NO levels could be reduced as a result of consumption by the elevated ROS; alternatively, NO generation could be decreased by reduced NO synthase (NOS) activity due to limitation of O_2 substrate availability in hypoxia. However, if this was the case, it would be expected that administration of the substrate L-arginine would not be effective in restoring NO levels during hypoxia. The observation that administration of L-arginine and of sNO had the same effects, qualitatively and quantitatively, suggests that the decrease in microvascular NO levels is not the result of reduced NO synthesis, but of increased consumption by ROS (Steiner et al., 2002).

1.2 Alveolar hypoxia and mast cell activation

Mast cell degranulation is a necessary event which provides the chemotactic gradient for the increased leukocyte-endothelial adhesive interactions of hypoxia (Steiner et al., 2003). Prevention of mast cell degranulation with cromolyn, a mast cell stabilizer, attenuates all of the markers of inflammation (Shah et al., 2003; Steiner et al., 2003). The inflammatory cascade includes activation of the local renin-angiotensin system (RAS): the increased leukocyte-endothelial adherence and vascular permeability observed in skeletal muscle during alveolar hypoxia are attenuated by inhibition of angiotensin converting enzyme (ACE) and by blockade of angiotensin II (Ang II) receptors (Gonzalez et al., 2007b).

A series of observations suggests that the key initial event of the inflammatory response, the activation of mast cells, is not triggered by the reduced PO_2 of the environment surrounding the mast cells, but rather by an agent(s) released by alveolar macrophages into the circulation.

First, selective reduction of tissue microvascular PO_2 does not induce inflammation unless it is accompanied by alveolar hypoxia. Cremaster microvascular PO_2 ($CmvPO_2$), measured using a phosphorescence decay assay (Shah et al., 2003), was selectively reduced in rats breathing room air. Cremaster hypoxia was induced either by mechanical restriction of cremaster blood flow (Shah et al., 2003), or by *in vivo* equilibration of the cremaster with 5% CO_2 /95% N_2 (Dix et al., 2003; Shah et al., 2003) in the presence of normal systemic arterial and alveolar PO_2 . Although $CmvPO_2$ decreased to levels comparable to those

seen in rats breathing 10% O₂, neither of these interventions produced mast cell degranulation or leukocyte-endothelial adherence in cremaster post-capillary venules. On the other hand, cremaster mast cell degranulation and leukocyte adherence occurred when the animals breathed 10% O₂ and *CmvPO₂* was maintained at a higher than normal level (Dix et al., 2003; Shah et al., 2003). One possible explanation for these results, among other alternatives, is that mast cell degranulation is triggered by an agent released from a distant site.

Second, if a putative mediator released from a distant site is transported by the systemic circulation, it would be expected that plasma obtained from hypoxic animals would elicit inflammation in normoxic tissues. Plasma obtained from conscious rats breathing 10% O₂ for 5 min produced mast cell degranulation, leukocyte-endothelial adherence, and extravasation of albumin when applied to the cremaster muscle of normoxic rats (Orth et al., 2005). The inflammatory effect is specific for hypoxic rat plasma since plasma from normoxic animals did not exert any effect on the cremaster microcirculation. The inflammation is not triggered by mediators released from activated mast cells or adherent leukocytes into the plasma of the donor rat: pretreatment of the donor with cromolyn, which blocks alveolar hypoxia-induced mast cell degranulation and leukocyte adherence (Steiner et al., 2003), did not attenuate the response to hypoxic rat plasma. The inflammatory agent contained in hypoxic rat plasma is not originated in blood cells, since plasma separated from blood equilibrated *in vitro* with hypoxic gas mixtures did not produce inflammation (Orth et al., 2005).

1.3 Alveolar macrophage activation and systemic inflammation

Alveolar hypoxia induces lung inflammation, and alveolar macrophages play an important role in the modulation of this phenomenon. Rats breathing 10% O₂ for periods ranging from 1 to 8 h show extravasation of albumin and increased pulmonary expression of HIF-1 α , NF- κ B, and pro-inflammatory cytokines; these markers are attenuated by elimination of alveolar macrophages (Beck-Schimmer et al., 2001; Leeper-Woodford and Detmer, 1999; Madjdpour et al., 2003; VanOtteren et al., 1995). Hypoxia leads to upregulation of the expression of neurokinin-1 receptors in alveolar macrophages and epithelial cells (Zee et al., 2006). Activation of these receptors leads to inflammatory responses mediated by cytokines IL-1, IL-6, and TNF- α (Lotz et al., 1988; Zee et al., 2006). Furthermore, alveolar macrophages have been implicated in the synergistic effects of hypoxia on pathogen-induced lung inflammation (Agorreta et al., 2005; Vuichard et al., 2005).

In addition to the well known pulmonary effects of alveolar macrophage activation with hypoxia and other stimuli, there is mounting evidence that activation of alveolar macrophages has substantial extrapulmonary effects. An example is the systemic microvascular response to particulate matter inhalation. Epidemiological studies have demonstrated a correlation between environmental air pollution and cardiovascular morbidity (Simkhovich et al., 2008), and clinical and experimental studies have shown that phagocytosis of fine particles by alveolar macrophages leads to pulmonary inflammation with increased number of

activated alveolar macrophages (Tamagawa et al., 2008). This is accompanied by elevated levels of circulating cytokines, systemic inflammation, and microvascular endothelial dysfunction in the systemic circulation (Ishii et al., 2005; Nemmar et al., 2005; Nurkiewicz et al., 2006; van Eeden et al., 2001). It has been suggested that following phagocytosis of particulate matter, cytokines released by activated alveolar macrophages act on the bone marrow to mobilize platelets and leukocytes which stimulate the release of acute phase proteins and lead to systemic inflammation (Fujii et al., 2002).

Alveolar macrophages are necessary for the inflammation of alveolar hypoxia which was demonstrated by three lines of evidence (Gonzalez et al., 2007a): first, depletion of alveolar macrophages by tracheal instillation of clodronate-containing liposomes blocked the mast cell degranulation, the increased leukocyte-endothelial adherence and the extravasation of albumin that follows alveolar hypoxia in intact rats. Second, plasma obtained from hypoxic, alveolar macrophage-depleted rats did not elicit inflammation when applied to the normoxic cremaster; third, supernatant of primary cultures from alveolar macrophages exposed to 10% O₂ induced mast cell degranulation and leukocyte-endothelial adherence when applied topically onto the normoxic cremaster. A non-specific effect was ruled out by the observation that supernatant of alveolar macrophages cultured in normoxia failed to exert any inflammation on the cremaster microcirculation. The inflammation initiated by hypoxic alveolar macrophage supernatant shares common pathways with that

secondary to alveolar hypoxia in intact animals, since both are attenuated by blockade of the RAS (Gonzalez et al., 2007a; Gonzalez et al., 2007b).

1.4 Mast cell activation and RAS

Plasma from hypoxic rats produces not only increased venular leukocyte-endothelial interactions and mast cell activation, but also produced local arteriolar vasoconstriction in the normoxic cremaster (Gonzalez et al., 2007a; Orth et al., 2005). Alveolar hypoxia, induced by 10% O₂ breathing, produced an initial arteriolar vasoconstriction in skeletal muscle which is followed by vasodilation (Orth et al., 2005). On the other hand, non-selective Ang II antagonists and ACE inhibitors attenuate the inflammation induced by alveolar hypoxia in intact rats (Gonzalez et al., 2007b). These results suggest a participation of the RAS in the inflammatory cascade initiated by alveolar hypoxia. In addition to the circulating RAS, local tissue RAS plays an important role in several pathophysiological processes, including cardiovascular inflammation (Paul et al., 2006; Peters and Unger, 2007; Sironi et al., 2005). Ang II is a potent vasoconstrictor implicated in inflammatory processes in the cardiovascular system (Carey and Siragy, 2003; Cheng et al., 2005; Ferrario and Strawn, 2006) which can cause increased leukocyte-endothelial interactions and leukocyte recruitment, increased vascular permeability, and eventually tissue remodeling (Cheng et al., 2005; Strawn and Ferrario, 2002; Weir and Dzau, 1999). Similar to systemic hypoxia, Ang II stimulates leukocyte-endothelial interactions in postcapillary venules via

generation of reactive O₂ species (Suzuki et al., 2006); these effects can be blocked by antioxidant administration (Wood et al., 1999a). The inflammatory response to alveolar hypoxia, as well as the inflammation produced by plasma from hypoxic rats, can be blocked by ACE inhibitor, and by Ang II receptor blocker (Gonzalez et al., 2007b).

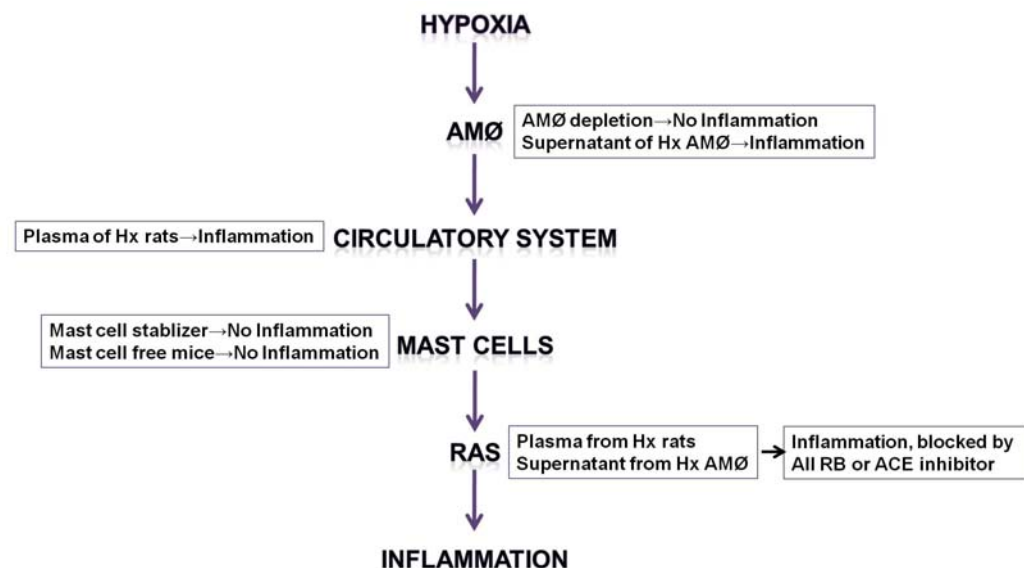
The following evidences suggest that mast cells are involved in RAS activation: Cromolyn, a mast cell stabilizer, abolish the inflammatory response of hypoxic rat plasma on normoxic cremaster, which suggests that mast cell activation is necessary for the inflammation originated by hypoxic rat plasma (Gonzalez et al., 2007b). The inflammation produced by hypoxic rat plasma via mast cell is blocked by Ang II receptor blockade and by ACE inhibitors (Gonzalez et al., 2007b); on the other hand, topical Ang II produces inflammation in the normoxic cremaster without activating mast cells; and the inflammatory effects of Ang II are not blocked by cromolyn (Gonzalez et al., 2007b). Finally, the inflammation produced by stimulation of mast cells with compound 48/80, a mast cell secretagogue, is attenuated by ACE inhibition and Ang II receptor blockade. The result obtained from intact animals suggest that the activation of RAS occurs downstream of mast cell degranulation.

The exact mechanisms underlying the activation of RAS by mast cells remain uncertain. Mast cells from some species contain chymases which act as ACE to convert Ang I to Ang II; moreover, renin contained in mast cells may initiate the RAS cascade. Consistent with this, a recent study demonstrated that renin

released from cardiac mast cell activates the RAS during ischemia/reperfusion (Silver et al., 2004).

In summary, previous data from our laboratory support the idea that a putative mediator released from alveolar macrophages during alveolar hypoxia activates perivascular mast cells, which, in turn initiate the inflammatory cascade by activating the RAS system.

The diagram below represents schematically the status of our understanding of this subject at the moment that the research described here was initiated:



AMØ: Alveolar macrophage

Hx: Hypoxia

All RB: Angiotensin II receptor blocker

ACE: Angiotensin Converting Enzyme

CHAPTER 2

General methods

2.1 Bronchoalveolar lavage (BAL) and culture of alveolar macrophage (Chao et al., 2009a; Gonzalez et al., 2007a)

Sprague-Dawley rats (300–350g) were anesthetized with pentobarbital sodium (40 mg/kg i.p.). A PE-50 catheter was placed in the jugular vein, a tracheotomy was performed, and a PE-240 catheter was inserted in the trachea. An overdose of sodium pentobarbital (150 mg/kg i.v.) was administered to euthanize the animals. Phosphate-buffered saline (PBS, 10 ml) was injected slowly into the lungs; the liquid was then removed and transferred into a plastic tube. This operation was repeated 10 times. The fluid collected was centrifuged at 1,500 rpm for 10 min. The cell pellets were pooled and resuspended in 2 ml of Dubelcco's Modified Eagle's Medium (DMEM) with 10% serum containing penicillin (100 U/ml) and streptomycin (100 µg/ml). A small sample of the suspension was obtained for cell counting in a hemocytometer.

Approximately 7 million alveolar macrophages are typically obtained from each rat using this procedure. Excluding variable amounts of red blood cells, which were eventually washed out (see below), the cells recovered were essentially all alveolar macrophages. The cells were plated in a T-25 sterile flask at 37°C and equilibrated with 5% CO₂ in air for 45 min. At this moment, the alveolar macrophages, but not the red blood cells, were firmly adherent to the flask. The

supernatant was discarded with a pipette, which resulted in elimination of red blood cells. The adherent alveolar macrophages were washed twice with 2 ml serum-free DMEM. After washing, 2 ml of serum-free DMEM were added and the flask was placed in a cell culture incubator at 37°C.

The cell culture was equilibrated with humidified gas mixtures via an 18-gauge needle placed on the flask's cap and connected to the gas source. Care was taken to place the tip of the needle a few mm above the surface of the medium so as not to disturb the culture. Incubation with humidified gas mixtures with nominal concentrations of O₂ (all gases had 5% CO₂ with the balance made up with N₂) was maintained for 30 min. Using this system, stable PO₂ values are reached within 2 minutes. After 30 min of equilibration, the supernatant was either used immediately or removed and frozen at -80°C. In the latter case, the supernatant was thawed on the day of the experiment. In either case, when the 30 minutes equilibration with the test gas mixture was finished and the supernatant removed, 0.4 % Trypan Blue (0.2 ml) was added to the culture flask. Within 30 minutes of addition of Trypan Blue, photographs were obtained from 5 different areas of the culture, containing approximately 200 cells each. The cells were counted to estimate cell viability as determined by exclusion of Trypan Blue.

2.2 Peritoneal lavage and culture of isolated peritoneal mast cells (Chao et al., 2009a; Poole and Zetter, 1983)

Peritoneal mast cells were harvested by lavage of the peritoneal cavity. Sprague-Dawley rats (250–300g) were anesthetized with ketamine 45 mg/kg i.m., atropine 0.4 mg/kg i.m., and a PE-50 catheter was placed in the jugular vein. Twenty ml of DMEM with 10% serum containing penicillin (100 U/ml), streptomycin (100 µg / ml) and heparin (5 U/ml) were injected into the peritoneal cavity, followed by gentle massage and recovery of fluid into cooled polypropylene tubes. Usually, cells obtained from 2 rats were pooled and used for one primary culture. Once the lavage was completed, the rats were euthanized with an overdose of sodium pentobarbital (150 mg/kg i.v.).

The cells were centrifuged at 400g for 15 min at room temperature and washed twice with serum-free DMEM. Cell pellets were resuspended in 0.75 ml serum-free DMEM, and macrophages were separated from mast cells by differential centrifugation using a Percoll solution as described before. Mast cells isolated by this procedure exceed 95% in purity.

The separated mast cells were resuspended in 2ml of DMEM with 10% serum containing penicillin (100 U/ml), streptomycin (100 µg/ml) and plated in a T-25 sterile flask at 37°C in 10% O₂-5% CO₂ - 85% N₂ for 45 min. The mast cells were collected by centrifugation at 3000 rpm for 2 minutes. The supernatant was discarded with a pipette and resuspended in 2 ml of serum-free DMEM. The cell culture was equilibrated with humidified gas mixtures (10% O₂-5% CO₂-85% N₂) via an 18-gauge needle placed on the flask's cap. Forty five min later, aliquots containing 0.4×10⁶ cells each were placed into centrifuge tubes and centrifuged

at 3000 rpm for 2 minutes. The supernatants were discarded and replaced with 0.4ml medium, plasma or alveolar macrophage supernatant, depending on the experimental protocol. The cells were incubated for 20 minutes and then centrifuged at 3000 rpm for 2 minutes. The cells were resuspended in 40µl of ruthenium red (50 µg/ml) to yield cell concentration of 10^7 cell/ml. The percentage of degranulated mast cells was checked with cell smear in a Nikon TE2000-S microscope. Photographs were obtained from 5 different areas of the slide, containing approximately 20 cells each.

2.3 Selective depletion of alveolar macrophages (Gonzalez et al., 2007a; Van Rooijen and Sanders, 1994)

Tracheal instillation of clodronate-containing liposomes was used to deplete alveolar macrophages in rats. Liposomes were composed of phosphatidylcholine and cholesterol in a 6-to-1 molar ratio and contained either clodronate or phosphate-buffered saline (PBS). Clodronate was a gift of Roche Diagnostics (Mannheim, Germany). It was encapsulated in liposomes as described previously.

Male Sprague-Dawley rats (300–350 g) were anesthetized with sodium pentobarbital (40 mg/kg i.p.), and the trachea was exposed with a midline incision. The animals were randomly assigned to either an alveolar macrophage depletion group, which received liposomes containing clodronate, or a control group, in which the rats were administered PBS-containing liposomes. The

animals were positioned at an angle of 30° head up, and 350 µl of a solution containing 250 mg clodronate liposomes/ml was injected between the tracheal cartilages with a 28-gauge needle. The skin incision was sutured, and the animals remained in the tilted position for 30 min, after which they were returned to their cages. The animals recovered uneventfully, and experiments were carried out 4 days after liposome administration.

The effectiveness of clodronate-containing liposomes was estimated by measuring the number of alveolar macrophages recovered in BAL described in section 1. The animals were studied four days after liposome administration. At the end of the experiment, the animals were anesthetized with sodium pentobarbital 40 mg/kg i.v, and 5 ml of blood removed into a heparinized syringe. Plasma was obtained by centrifugation and used for the mast cell immersion experiments. After blood removal, the animals were euthanized with 150mg/kg sodium pentobarbital, and BAL was carried as described above. Alveolar macrophages recovered in BAL were counted in a hemocytometer.

2.4 Exposure of conscious rats to hypoxia (Gonzalez et al., 2007a)

Three days after injection of clodronate or PBS liposomes the rats were anesthetized with pentobarbital sodium (40 mg/kg i.p.). PE-50 catheters were placed in the carotid artery and external jugular vein, tunneled subcutaneously, exteriorized at the back of the neck, and flame sealed. Twenty four hours later, the rats were placed into a Lucite chamber in which 10% O₂-90% N₂ was

circulated. Before and at 5, 30 and 60 min of exposure to hypoxia, a 1-ml blood sample was obtained in a 5-ml syringe coated with heparin (1,000 USP units/ml), and the blood was centrifuged at 3000 rpm for 10 min. Plasma was separated and stored at -80°C. Withdrawn blood was replaced after sampling by blood obtained from donor rats.

2.5 Intravital Microscopy (Chao et al., 2009a; Wood et al., 1999a)

Male Sprague-Dawley rats (250–300 g) were anesthetized with ketamine 45 mg/kg, atropine 0.4 mg/kg i.m. PE-50 catheters were placed in the jugular vein and carotid artery for injection of solutions and measurement of arterial blood pressure. The abdomen was opened via a midline incision and the ileo-cecal portion of the intestine was gently drawn out, exteriorized, and mounted on a transparent plastic stage. The intestinal loop was covered with Saran wrap to prevent drying of the tissue and to minimize the effect of ambient oxygen on the mesenteric microcirculation. The Saran wrap cover was briefly lifted when solutions were applied topically to the mesentery. The animals were covered with a thermal blanket to maintain rectal temperature at 37°C.

The mesentery microcirculation was observed by transillumination using an inverted microscope. Color images of the microcirculation were shown in a video monitor by a camera mounted on the microscope. The images were recorded on a videocassette recorder with a time-date generator for off-camera analysis. Centerline red blood cell velocity was measured using an optical Doppler

velocimeter. Average red blood cell velocity was calculated as centerline velocity/1.6. Venular diameter was measured using a video caliper. Single unbranched post-capillary venules, with a diameter of 20-40 μm , length of ~ 200 μm , less than 3 adherent leukocytes /100 μm , and a steady venular blood velocity of at least 2 mm/sec were selected for observation in this study. Adherent leukocytes were defined as those remaining stationary for 30 sec. Leukocyte endothelial adherence (LEA) was expressed as number of adherent leukocytes / 100 μm of venule length. Ruthenium red (5 mg/100 ml) was applied topically to document *in vivo* mast cell degranulation. The mast cell images were converted to digitized grayscales and phase-inverted. The relative light intensity of each mast cell within the field of view was measured using the AnaliSYS Software System, and the extent of ruthenium red uptake was estimated from the gray scale intensity value and expressed in arbitrary units. At least 5 mast cells were analyzed in each field of observation. At the end of the experiment, the rats were killed with an overdose of sodium pentobarbital, 150 mg/kg i.v.

2.6 Statistics

Data are means \pm SEM. Each preparation served as its own control, with the data obtained after a given treatment compared with that obtained during the control period. Significance was established using a t test for paired values. Intergroup comparisons were made with a one way ANOVA with the Bonferroni correction for multiple comparisons.

CHAPTER 3

Interaction between alveolar macrophages and mast cells in the inflammatory response induced by hypoxia

3.1 Introduction

Alveolar hypoxia, induced by reduction of inspired PO_2 , initiates a rapid and widespread inflammatory response in mesentery (Wood et al., 1999b), skeletal muscle (Dix et al., 2003; Shah et al., 2003) and brain (Mc Donald and Wood, 2003) of rats. The inflammation is characterized by increased microvascular levels of ROS (Wood et al., 1999a), perivascular mast cell degranulation (Dix et al., 2003; Shah et al., 2003; Steiner et al., 2003), increased leukocyte-endothelial adhesive interactions (Wood et al., 1999b), and extravasation of albumin (Wood et al., 2000).

Studies in the cremaster microcirculation suggest that the inflammation elicited by alveolar hypoxia is not triggered by the reduction of cremaster PO_2 , but rather by a mediator released from a distant site and transported by the circulation. This idea is supported by two lines of evidence: first, selective reduction of cremaster PO_2 does not produce mast cell degranulation and inflammation in the cremaster microcirculation unless alveolar PO_2 is also reduced (Dix et al., 2003; Shah et al., 2003); second, plasma obtained from hypoxic rats applied to the normoxic cremaster produces an inflammatory response similar to that elicited by alveolar

hypoxia (Orth et al., 2005). The response to hypoxic rat plasma is not due to inflammatory mediators released into plasma by activated mast cells or adherent leukocytes of the donor rat; furthermore, the agent(s) responsible for the inflammation is not generated by blood cells (Orth et al., 2005). Further investigation showed that the putative mediator activates mast cells and initiates an inflammatory cascade that includes activation of the renin-angiotensin system (RAS) (Gonzalez et al., 2007b).

A role for alveolar macrophages as a source of the putative mediator is supported by the findings that alveolar macrophage depletion *in vivo* attenuates the inflammatory response to alveolar hypoxia, and that supernatant of alveolar macrophages cultured in hypoxia induces mast cell degranulation and inflammation in normoxic cremaster muscle (Gonzalez et al., 2007a). Similar to the inflammation elicited by alveolar hypoxia (Gonzalez et al., 2007b), the response to alveolar macrophage supernatant was abrogated by pretreatment with cromolyn, a mast cell stabilizer, and with Ang II receptor antagonists (Gonzalez et al., 2007a).

The present experiments were designed to provide evidence of a direct link between the activation of alveolar macrophages by the reduced alveolar PO₂ and the degranulation of tissue mast cells which initiates the inflammation of hypoxia. We reasoned that if an alveolar macrophage-borne mediator(s) triggers the inflammation by activating mast cells, reduced PO₂ would not directly activate primary mast cell cultures, but, on the other hand, these cells would degranulate

when placed in contact with supernatant of hypoxic alveolar macrophages. Furthermore, we hypothesized that hypoxia would not directly activate isolated resident tissue macrophages, and that mast cells exposed to supernatant of resident tissue macrophages cultured in hypoxia would not undergo degranulation. The results confirm our hypothesis that the inflammation of alveolar hypoxia is triggered by a mediator(s) released by activated alveolar macrophages, and rule out possible contributions of local hypoxia and of resident tissue macrophages in the initiation of the inflammation. A mast cell secretagogue, monocyte-chemoattractant protein-1, was identified as a possible candidate for the putative mediator of hypoxia-induced systemic inflammation.

The phenomenon described here highlights a systemic effect of alveolar macrophage activation, and could provide a possible pathogenic mechanism to explain the systemic consequences of conditions associated with reduced alveolar PO₂.

3.2 Methods

The general methods and techniques utilized are described in detail in chapter 2.

The experiments described below included *in vivo* studies in which supernatant of primary cell cultures as well as pharmacological agents were applied topically

to the mesentery of normoxic rats, and *in vitro* studies carried out in primary cell cultures.

Alveolar macrophages in an intact animal are normally exposed to a higher PO_2 than peritoneal macrophages or peritoneal mast cells, and this difference subsists in conditions of hypoxia. Accordingly, humidified gas mixtures for the cell culture experiments were used with nominal concentrations of 21, 10, and 0% O_2 . All gas mixtures contained 5% CO_2 with the balance made up with N_2 . These gas mixtures provided PO_2 values that encompass the range observed *in vivo*, from normoxic to hypoxic conditions, in the cell types studied here. The PO_2 attained in the liquid phase during gaseous equilibration in an open system depends on the efficacy of the equilibration system. In the present experiments, the cell cultures, placed in an incubator at 37°C, were gassed via a needle inserted in the cap of the culture dish and connected to the gas source. Care was taken to place the tip of the needle a few mm above the surface of the culture medium so as to not disturb the culture. In order to directly determine the efficacy of the equilibrating system, PO_2 of the medium was measured directly in some of the experiments using a phosphorescence decay method (Lo et al., 1996). This technique is currently employed in our laboratory to measure microvascular PO_2 of intact animals (Dix et al., 2003; Shah et al., 2003). The actual supernatant PO_2 values were (Torr, mean \pm SEM): 0% O_2 : 4.8 ± 0.8 , and 10 % O_2 : 65.3 ± 0.9 . These values were attained within 2-3 minutes of equilibration. Exposure to 21% O_2 produced PO_2 values outside the range of the method (>100 Torr). Alveolar

macrophages may be exposed *in vivo* to PO_2 of ~ 65 Torr in moderate cases of hypoxia. In contrast, peritoneal macrophages and mast cells exposed to 10% O_2 will be in an environment which is at the high end of the PO_2 values observed *in vivo*. On the other hand, PO_2 of ~ 5 Torr is the value to which peritoneal macrophages, mast cells and other systemic tissue cells would be exposed in a rat breathing 10% O_2 (Dix et al., 2003; Shah et al., 2003).

3.2.1 Measurement of H_2O_2 in supernatant of alveolar and peritoneal macrophages

An electrochemical detection system (Apollo 4000, World Precision Instruments) was used to determine H_2O_2 concentration in 0.3 ml aliquots of supernatant of alveolar and peritoneal macrophages isolated and cultured on the day of measurement. Measurements were carried out in samples removed at 15 min intervals using an H_2O_2 -sensitive electrode at $37^\circ C$. After measurement, the sample was returned to the culture dish. When the experiments were finished, 2 ml of a solution of 0.4% Trypan Blue was added to the culture and mixed for 2 minutes. Photographs of 5 different areas of the culture, containing approximately 200 cells each, were obtained within 30 min of Trypan Blue addition. Cell viability was expressed as the percentage of cells excluding Trypan Blue.

3.2.2 Measurement of inflammatory mediators

The effect of hypoxia on supernatant levels of several cytokines and chemokines was investigated by initially screening cytokine and chemokine levels using a multianalyte ELISA Array (SABiosciences Corporation). Agents investigated in the initial screen included: Interleukins-1 β , -4, -6, -10, -12, -17 α ; IFN- γ , TNF- α , Transforming Growth Factor-B1, Monocyte Chemoattractant Protein -1 (MCP-1), and Macrophage Inflammatory Protein (MIP) -1 α and -1 β . The initial screen was followed by individual assay (Single Analyte ELISA, SABiosciences Corporation) to determine levels of MCP-1 in supernatant of alveolar macrophages, peritoneal macrophages, and peritoneal mast cells.

3.3 Results

3.3.1 EXPERIMENTS IN THE MESENTERIC MICROCIRCULATION

All experiments of this series had the same format: after a 30 min control period, ~0.5 ml of supernatant or of a pharmacological agent was distributed evenly over the mesentery. The microcirculation was observed for an additional 30 min. The supernatants used had been frozen and were thawed at 37°C immediately before use.

Effect of alveolar or peritoneal macrophage supernatant applied on the mesentery of normoxic rats

Supernatant of alveolar macrophages which had been equilibrated with 10% O₂ produced mast cell degranulation and leukocyte-endothelial adherence (Figure 1A) (N = number of rats studied = 5). In contrast, neither supernatant from alveolar macrophages equilibrated with 21 % O₂ (Figure 1B, N=5) or from peritoneal macrophages equilibrated with 0% O₂, (Figure 1C, N=5) produced mast cell degranulation or leukocyte-endothelial adherence.

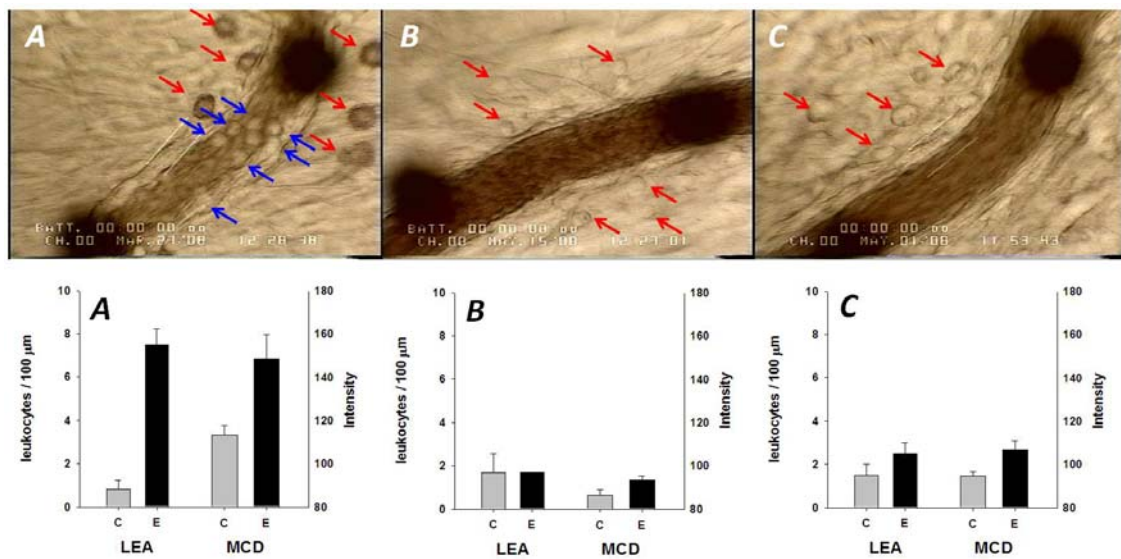


Figure 1: Effect of topical application of AMØ and PMØ supernatant on the mesentery

Top: Representative microphotographs of the mesenteric microcirculation after topical application of supernatant hypoxic alveolar macrophages (A), normoxic alveolar macrophages (B), and hypoxic peritoneal macrophages (C). The large dots are used to align the optical Doppler velocimeter and occasionally they are moved to obtain a better image of the leukocyte-endothelial interface for photographs. The red arrows point to the mast cells; the blue arrows identify adherent leukocytes. Bottom: average values of leukocyte-endothelial adherence (LEA) as assessed by number of leukocytes /100 µm, and intensity of mast cell degranulation (MCD) in arbitrary units. C and E: mean ± SEM of values at the end of the control and the experimental period, respectively. * P<0.01 vs. corresponding normoxic control N=number of rats studied = 5 in each group.

3.3.2 EXPERIMENTS IN PRIMARY CELL CULTURES

H₂O₂ release by isolated alveolar and peritoneal macrophages

All the experiments in this series had the same format: after a 45 minute period of equilibration with 21% O₂ for alveolar and 10% O₂ for peritoneal macrophages, the cultures were equilibrated for 60 min with the experimental gas mixture. Samples for supernatant H₂O₂ concentration measurement were obtained at the end of the normoxic control period and every 15 min thereafter.

Table 1 shows the changes in supernatant H₂O₂ concentration with respect to their respective controls in the different groups. Under control conditions (21% O₂), alveolar macrophage supernatant H₂O₂ concentration averaged 0.23 ± 0.05 nM/10⁶ cells, without significant differences among groups. Continuation of exposure to 21% O₂ for one additional hour resulted in a gradual decrease in H₂O₂ concentration of the supernatant. Hypoxia (nominal 10% O₂ and 0% O₂) produced a significant net increase in alveolar macrophage supernatant H₂O₂ concentration. The increase was significantly higher when the nominal O₂ concentration of the equilibrating gas mixture was 0% (actual PO₂ ~ 5 Torr) than when equilibration was carried out with 10% O₂ (actual PO₂ ~ 65 Torr). The increase was transitory, reaching a peak at 15 min of equilibration, and gradually returning towards control. The increase in supernatant H₂O₂ produced by equilibration with 10% O₂ was blocked by pretreatment with PEG catalase. Viability of alveolar macrophages, assessed by Trypan Blue exclusion at the end

of the experiments, was higher than 95% and was not influenced by the PO_2 , even when it reached values as low as ~ 5 Torr.

Table 1: Effects of hypoxia on H_2O_2 release by alveolar and peritoneal macrophages

Cell type	% O_2	Supernatant $\Delta[H_2O_2]$ (nM/ 10^6 cell)					No. of cultures
		15 min	30 min	45 min	60 min	%viability	
Alveolar macrophages	21	-0.03 ± 0.02	-0.11 $\pm 0.03^*$	-0.12 $\pm 0.03^*$	-0.09 ± 0.04	95.8 \pm 0.4	5
	10	0.28 $\pm 0.07^*$	0.09 ± 0.03	-0.01 ± 0.03	-0.06 ± 0.04	96.9 \pm 0.3	5
	0	1.05 $\pm 0.13^*$	-0.03 ± -0.02	-0.02 ± 0.02	-0.02 ± 0.02	98.6 \pm 0.6	5
	10 + CAT	0.02 ± 0.01	0.02 ± 0.02	0.00 ± 0.02	-0.01 ± 0.02	96.9 \pm 0.5	5
Peritoneal macrophages	10	-0.02 ± 0.01	-0.02 ± 0.02	-0.01 ± 0.02	-0.02 ± 0.02	95.9 \pm 0.9	5
	0	0.01 ± 0.01	0.01 ± 0.01	-0.01 ± 0.01	0.01 ± 0.02	95.2 \pm 0.5	5

Changes in the concentration of H_2O_2 with respect to the control: each value (N=5) is the average of the value at a given time minus the corresponding control. * $P < 0.05$ vs. corresponding normoxic control.

Average peritoneal macrophage supernatant H_2O_2 concentration under control conditions (10% O_2) was 0.31 ± 0.05 nM/ 10^6 cells, which was not significantly different from that observed in alveolar macrophages under control conditions (21% O_2). However, in contrast with alveolar macrophages, equilibration of peritoneal macrophages with 0% O_2 was not accompanied by H_2O_2 release into

the supernatant. Cell viability at the end of 1 hour of equilibration was also approximately 95%, and was not influenced by the supernatant PO_2 .

Because H_2O_2 activates mast cells and induces inflammation, additional experiments in the mesenteric microcirculation were carried out to determine whether the small amount of H_2O_2 present in the alveolar macrophage supernatant after 30 minutes of hypoxia could be responsible for the inflammatory response illustrated in Figure 1 A, where supernatant of alveolar macrophages equilibrated with 10% O_2 was applied topically on the mesentery. Topical application of a solution of 10 nM H_2O_2 , a concentration over 5 fold higher than that observed in the alveolar macrophage supernatant at 30 minutes of 10% O_2 equilibration, did not increase leukocyte-endothelial adherence or mast cell degranulation above that of the untreated controls (compare figures Figure 2A and 2B). Only when a solution with a concentration of 10 μ M was applied did leukocyte-endothelial adherence and mast cell degranulation become evident (Figure 2C, N=5).

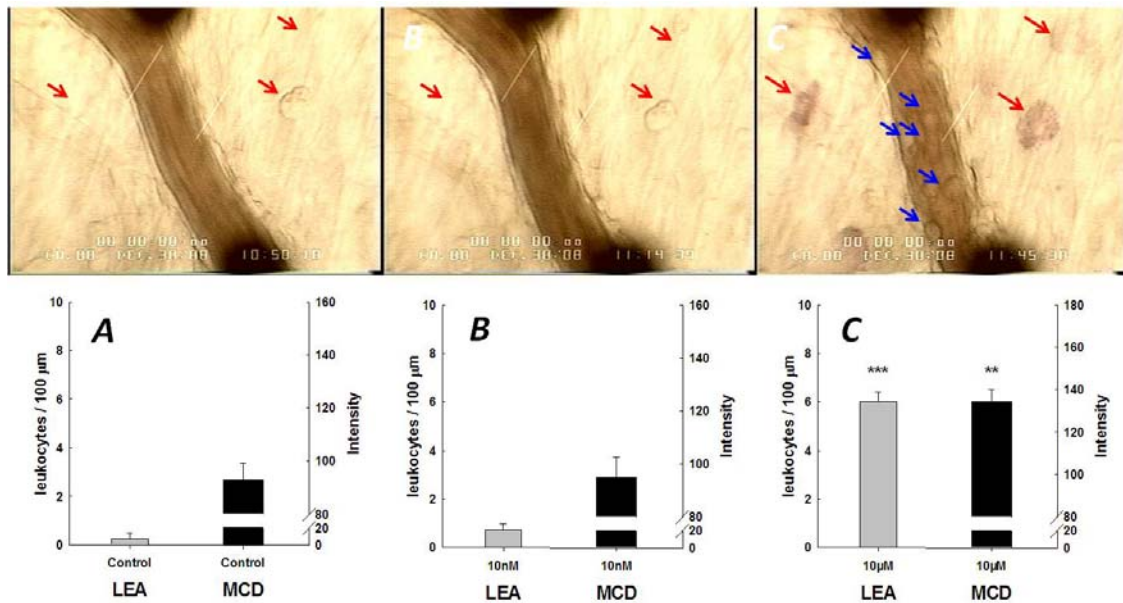


Figure 2: Effect of topical H₂O₂ on the normoxic mesentery

Top: representative microphotographs of the mesenteric microcirculation illustrating the effect of topical application of H₂O₂. A: untreated; B: after application of 10 nM H₂O₂ and C: after application of 10 μM H₂O₂. Since H₂O₂ bleaches ruthenium red, the photographs were taken after the H₂O₂ was washed out from the surface, after which ruthenium red was applied. Bottom: average values of LEA as assessed by number of leukocytes /100 μm, and intensity of MCD in arbitrary units. * P<0.01 vs. corresponding normoxic control. N=5 in all cases.

Effect of hypoxia on peritoneal mast cell degranulation

Fresh peritoneal mast cell cultures (N = number of cell cultures studied = 5, Figure 3) were successively exposed to gas mixtures with 10 and 0% O₂, and 0% O₂ plus C48/80. After 30 min of exposure to each condition, a small sample (5-10 μl, ~20,000 cells / μl) was removed and mixed with an equal volume of a solution of ruthenium red to assess mast cell degranulation. Exposure of peritoneal mast cells to 0% O₂ did not significantly increase uptake of ruthenium red from the levels observed during normoxic equilibration (compare Figure 3A and 3B).

Addition of C48/80 during hypoxia to cells obtained from the same cultures induced complete mast cell degranulation (Figure 3C), indicating that the hypoxic mast cells could respond normally to a general secretagogue.

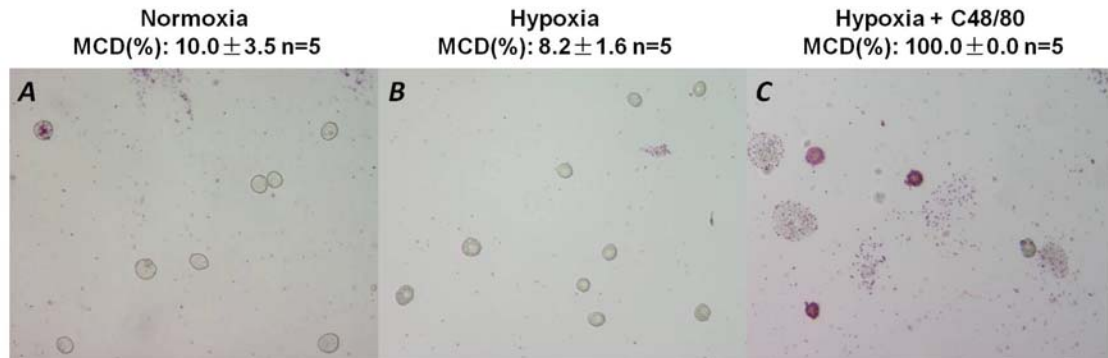


Figure 3: Effect of hypoxia on mast cell degranulation

Representative microphotographs of primary mast cell cultures. The cells represented in photographs A-C were obtained from the same primary culture. The data on top of each photograph is the mean \pm SEM of the percent of MCD in 5 separate experiments.

Interactions between isolated mast cells, alveolar macrophages and peritoneal macrophages

In these series, mast cells cultured in 10% O₂ were centrifuged, and the pellet suspended in supernatant removed from fresh primary cell cultures of alveolar or peritoneal macrophages. The supernatants contained no macrophages. The mast cells resuspended in macrophage supernatant were equilibrated with 10% O₂ in all cases.

Suspension of isolated mast cells in supernatant of alveolar macrophages which had been cultured in normoxia did not induce mast cell degranulation

(Figure 4A; N = number of cell cultures studied = 5); on the other hand, suspension in supernatant of alveolar macrophages cultured in hypoxia (10% O₂) significantly increased mast cell degranulation (Figure 4B, N=5). In contrast, suspension of mast cells in supernatant of peritoneal macrophages which had been incubated with 0% O₂, failed to produce mast cell degranulation (Figure 4C, N=5).

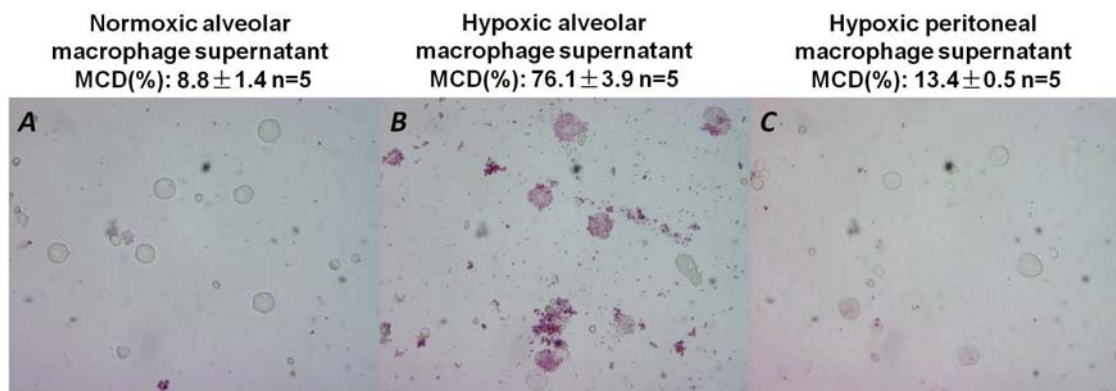


Figure 4: Effect of immersion of mast cells in supernatant of alveolar or peritoneal macrophages

Mast cells obtained for the same culture were immersed in supernatant of alveolar macrophages that had been equilibrated with 21% O₂ (A), 10% O₂ (B), or peritoneal macrophages equilibrated with 0% O₂. The supernatants contained no macrophages and after mast cell immersion were equilibrated with 10% O₂. The data on top of each photograph is the mean \pm SEM of the percent of degranulated mast cells in 5 separate experiments.

Effects of hypoxia on selected cytokine levels

Of all the agents screened in the supernatant of alveolar macrophages using a multi-analyte ELISA array (IL-1 β , -4, -6, -10, -12 and 17 α , IFN- γ , MCP-1, TNF- α , and MIP-1 α and -1 β), only MCP-1 showed a marked increase with hypoxia within the time frame of these experiments. Subsequent individual ELISA assays using

a specific antibody showed that MCP-1 increases almost four-fold in the supernatant of alveolar macrophages exposed to hypoxia, compared to the normoxic control (Figure 5). In contrast, equilibration with nominal 0% O₂ does not elicit release of MCP-1 from peritoneal macrophages or mast cells (Figure 5).

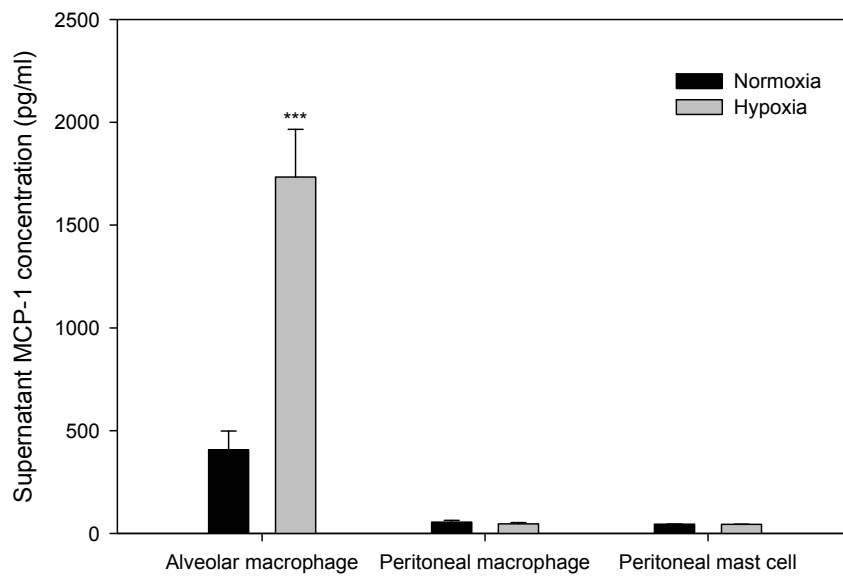


Figure 5: Effect of hypoxia on the release of MCP-1 by alveolar macrophages.

Supernatant monocyte chemoattractant protein-1 concentration of primary cultures of alveolar macrophages, peritoneal macrophages, and peritoneal mast cells, exposed to 30 min of hypoxia or normoxia. * $P < 0.01$ vs. corresponding normoxic control. $N = 5$ in each case.

3.4 Discussion

The central findings of these experiments are the following: 1. Supernatant of hypoxic alveolar macrophages elicited inflammation in the normoxic mesentery, while supernatant of peritoneal macrophages equilibrated with even lower PO₂ values had no inflammatory effect. 2. Hypoxia activated primary cultures of

alveolar, but not of peritoneal macrophages. 3. Mast cells in primary cultures did not degranulate when exposed to hypoxia; however, degranulation occurred when mast cells were placed in contact with supernatant of hypoxic alveolar macrophages, but not of hypoxic peritoneal macrophages. 4. Hypoxia induced release of MCP-1 from alveolar, but not peritoneal macrophages or mast cells.

These results demonstrate a direct link between alveolar macrophage activation by hypoxia and mast cell degranulation, and rule out possible roles of low tissue PO₂ and of resident tissue macrophages in the early phase of the inflammation of alveolar hypoxia. The results add a key piece of evidence that confirms our hypothesis that the inflammation produced by alveolar hypoxia is triggered by an alveolar macrophage-borne mediator(s) carried by the circulation. The release by alveolar macrophages exposed to hypoxia of a mast cell secretagogue, MCP-1, provides evidence concerning the nature of a possible mediator of inflammation.

Studies in the mesenteric microcirculation

The objectives of the *in vivo* studies in the mesenteric microcirculation were two: first, since the mast cells and resident tissue macrophages used in the present experiments were collected from the peritoneum, it was important to determine whether the responses of the primary cell cultures to the various interventions were similar to those observed *in vivo*. Peritoneal mast cells and macrophages were selected because they can be harvested with minimal

manipulation, and therefore their responses are least affected by the isolation procedures. The second objective of the *in vivo* studies was to demonstrate whether the response of the mesenteric microcirculation to hypoxic alveolar macrophage supernatant is similar to that observed previously in the cremaster. A difference in the responses of these two vascular beds would argue against our hypothesis that the widespread inflammation of alveolar hypoxia in the intact animal is triggered by a mediator released into the circulation by activated alveolar macrophages.

Topical application of supernatant of alveolar macrophages equilibrated with 10% O₂ produced essentially the same response in the mesentery as that observed previously in the cremaster microcirculation (Gonzalez et al., 2007b): mast cell degranulation, and increased leukocyte-endothelial adherence (Figure 1A).

An important new finding of these experiments was that supernatant of peritoneal macrophages exposed to a more severe level of hypoxia than alveolar macrophages did not initiate an inflammatory response in the mesentery (Figure 1C). This observation is consistent with the failure of hypoxia to induce a respiratory burst in cultures of peritoneal macrophages (Table 1). These findings, together with the absence of *in vivo* cremaster inflammation during selective cremaster hypoxia in the presence of normal alveolar PO₂ (Dix et al., 2003; Shah et al., 2003) indicate that resident tissue macrophages do not contribute to the initiation of the systemic inflammation of alveolar hypoxia.

In summary, the results show that the inflammatory response to supernatant of hypoxic alveolar macrophages has similar characteristics in the mesenteric and in the skeletal muscle microcirculations, and supports hypothesis that the widespread inflammation of alveolar hypoxia is the result of mast cell activation by an agent released by alveolar macrophages.

Studies in the primary cell cultures

Alveolar macrophages, but not peritoneal macrophages responded with a transitory release of H_2O_2 when exposed to hypoxia. The H_2O_2 release is a manifestation of the respiratory burst which occurs during macrophage activation (Ma et al., 2003) and is characterized by superoxide generation followed by dismutation to H_2O_2 . In addition to an important role in inactivation of phagocytosed pathogens, reactive O_2 species generated during the respiratory burst are thought to play a role in intracellular signal transduction (Gwinn and Vallyathan, 2006; Iles and Forman, 2002). In the present experiments, the amount of H_2O_2 released by alveolar macrophages was largest at the lowest PO_2 . The correlation between the magnitude of the respiratory burst and the severity of hypoxia in the presence of maintained cell viability indicates that this is a biological response of alveolar macrophages to reduced PO_2 . On the other hand, peritoneal macrophages exposed to the same PO_2 did not become activated. The dissimilar effects of hypoxia on activation of the two types of macrophages are paralleled by the different effects of their supernatants on mast cells: while supernatant of

hypoxic alveolar macrophages elicited degranulation of mast cells both *in vivo* (Figure 1A) and *in vitro* (Figure 4B), supernatant of hypoxic peritoneal macrophages exposed to much lower PO₂ had none of these effects (Figure 1C and 4C). Thus, evidence obtained both *in vivo* and *in vitro* points to a specific effect of reduced PO₂ on alveolar macrophages.

The discrepancy between alveolar and peritoneal macrophages in the response to hypoxia is a manifestation of the different characteristics of these two cell types. While both originate from a common precursor in the bone marrow, alveolar macrophages *in vivo* are normally exposed to a higher PO₂ than peritoneal macrophages. These different environments may in part determine their different metabolic patterns (Simon et al., 1977) and perhaps explain the dissimilar effects of hypoxia.

The present experiments clearly demonstrate a link between activation of alveolar macrophages by hypoxia and mast cell degranulation: degranulation did not occur when mast cells were directly exposed to hypoxia, but was observed when the mast cells were immersed in supernatant of alveolar macrophages that had been equilibrated in hypoxia. This shows that hypoxic alveolar macrophages release an agent which produces mast cell degranulation. This agent is not released by normoxic alveolar macrophages or by hypoxic peritoneal macrophages which show no evidence of activation. Our earlier *in vivo* finding that selective cremaster hypoxia does not induce mast cell degranulation in rats with normal alveolar PO₂ (Dix et al., 2003) agrees with these *in vitro* observations

and demonstrate that neither reduced local PO₂ nor activation of resident tissue macrophages participate in the systemic inflammation of hypoxia.

A key issue in this phenomenon is the nature of the mediator released by alveolar macrophages in response to hypoxia. There is evidence that alveolar hypoxia leads to pulmonary inflammation in rats (Madjdpour et al., 2003), and alveolar macrophages play an important role in this phenomenon. Hypoxia increases expression of TNF- α and MIP-1 β either in the lung or in isolated alveolar macrophages (Madjdpour et al., 2003; VanOtteren et al., 1995). In addition, hypoxia acts as a synergist in the interaction of several pathogens with alveolar macrophages (Agorreta et al., 2005; Leeper-Woodford and Detmer, 1999; Vuichard et al., 2005). However, while these responses may influence the development of pulmonary and systemic inflammation later on in the course of hypoxia, it is unlikely that the phenomenon described in the present experiments is a consequence of changes in gene expression. Given the rapid onset of the response to hypoxia, and the fact that the targets are the mast cells, the putative mediator of systemic inflammation is likely to be a mast cell secretagogue stored in alveolar macrophages. To provide additional information on this subject, several possible candidates were screened. MCP-1 was observed to increase significantly in the supernatant of alveolar macrophages exposed to hypoxia in the time frame used in these studies. Peritoneal macrophages or mast cells did not release MCP-1 when exposed to even lower PO₂. MCP-1, a chemokine of the CC family, fits the criteria for a putative mediator of hypoxia-induced

inflammation: MCP-1 induces chemotaxis of alveolar macrophages, mast cells, and human T-lymphocytes (de Boer et al., 2000). MCP-1 is released from alveolar macrophages *in vitro* in response to hypoxia and hypoxia/reoxygenation (McCourtie et al., 2008; Zhao et al., 2006), influences distal organ damage in hemorrhagic shock (Frink et al., 2007) and activates mast cells to elicit microvascular inflammation (Conti et al., 1995; Wan et al., 2003). Further studies are necessary to determine the mechanism underlying the release of MCP-1 by alveolar macrophages, the interaction of MCP-1 with mast cells, and whether other alveolar macrophage-borne agents participate in the activation of mast cells. Nevertheless, the demonstration of increased release of a mast cell secretagogue provides confirmation to our hypothesis that the systemic inflammation of hypoxia is initiated by an alveolar macrophage-borne mediator.

The present results highlight the role of alveolar macrophages in initiating an inflammatory response in the systemic microcirculation. Although most known functions of these cells take place within the lung, mounting evidence indicates that alveolar macrophage activation leads to systemic inflammation and microvascular function impairment. For example, following activation by phagocytosis of particulate matter, cytokines released by alveolar macrophages act on the bone marrow to mobilize platelets and leukocytes which stimulate the release of acute phase proteins and lead to systemic inflammation (Ishii et al., 2005; van Eeden et al., 2001). The phenomenon described in the present study

represents an example of systemic effects initiated by activation of alveolar macrophages by another type of stimulus, in this case, reduction of alveolar PO₂.

Several conditions featuring alveolar hypoxia are accompanied by systemic effects, and inflammation has been implicated as a causative or contributory factor in these systemic effects. These include the cachexia and muscle wasting (Agusti and Soriano, 2008; Wust and Degens, 2007) and the cardiovascular abnormalities (Eickhoff et al., 2008) of COPD, the suboptimal erythropoiesis of pulmonary fibrosis (Tsantes et al., 2003b) and the cardiovascular complications of sleep apnea (Jelic et al., 2008; Morgan, 2007). Conditions in which hypoxia develops rapidly are also accompanied by a systemic inflammatory component. For instance, pneumonia is accompanied by systemic inflammation, and elevated circulating inflammatory markers are predictors of subsequent mortality (Fernandez-Serrano et al., 2003; Yende et al., 2008). While these markers may in part reflect the response to bacterial infection, it is conceivable that the hypoxic environment in areas of lung consolidation may lead to activation of alveolar macrophages which contribute to the systemic inflammation.

Acute altitude illnesses represent another example of systemic inflammatory response to alveolar hypoxia. Rapid ascent to high altitude is increasingly frequent and may result in acute mountain sickness or high altitude cerebral edema (Basnyat and Murdoch, 2003). While the role of inflammation in these cases is still unclear, the presence of elevated levels of circulating and tissue inflammatory markers (Beidleman et al., 2006; Hartmann et al., 2000; Klausen et

al., 1997), and the effectiveness of dexamethasone, an anti-inflammatory steroid, in the treatment of acute mountain sickness and high altitude cerebral edema (Basnyat and Murdoch, 2003; Wright, 2006) suggest the contribution of an inflammatory component. Whether activation of alveolar macrophages plays a role in the pathogenesis of these and other conditions associated with alveolar hypoxia should be the subject of further research.

It is important to keep in mind that reduction of alveolar PO₂ in an intact organism is a complex stimulus which sets in motion responses with different time courses; accordingly, a number of mechanisms may participate at different moments in the course of hypoxia. For example, increased leukocyte-endothelial adhesive interactions in response to hypoxia have been demonstrated in isolated human umbilical veins, in which the phenomenon described here clearly does not occur (Michiels et al., 2000). However, the time course of the *in vitro* responses (hours vs minutes) is quite different from that described in the present experiments, suggesting that the underlying mechanisms are different from those described here.

In summary, the present study provides key evidence in support of our hypothesis that the inflammation of alveolar hypoxia is initiated by the release of a mediator from alveolar macrophages. This evidence includes the identification of a possible mediator of this phenomenon, a mast cell secretagogue produced by alveolar macrophages. Further research is necessary to determine whether other agents participate, and to develop tools to antagonize the mediator's

effects. This should help understand the possible contribution of this phenomenon to the pathogenesis of illnesses associated with reduced alveolar PO_2 , as well as its role in the overall strategies of adaptation of organisms to alveolar hypoxia.

CHAPTER 4

Monocyte Chemoattractant Protein-1 (MCP-1/CCL2) released from alveolar macrophages mediates the systemic inflammation of acute alveolar hypoxia

4.1 Introduction

The previous data from our lab suggest that the systemic inflammation elicited by alveolar hypoxia is not triggered by the reduction of peripheral tissue PO₂, but rather by a mediator(s) released from alveolar macrophages and transported by the circulation (Chao et al., 2009a; Chao et al., 2009b; Gonzalez et al., 2007a). The mediator activates local tissue mast cells which release inflammatory agents, including activation of the RAS (Gonzalez et al., 2007b), to initiate the systemic inflammation. Mast cells play a key event since the mast cell stabilizer cromolyn attenuates all of the markers of inflammation induced by alveolar hypoxia (Shah et al., 2003; Steiner et al., 2003). Alveolar macrophages are the origin of the mediator, which is supported by several lines of evidence: 1) Depletion of alveolar macrophages attenuates the systemic inflammatory response induced by alveolar hypoxia (Gonzalez et al., 2007a); 2) Plasma obtained from intact hypoxic rats, but not from alveolar macrophage-depleted hypoxic rats, produce an inflammatory response in normoxic cremaster (Gonzalez et al., 2007a). The effect of hypoxic rat plasma is not due to

inflammatory mediators released into plasma by activated mast cells or adherent leukocytes of the donor rat; furthermore, the agent(s) responsible for the inflammation is not generated by blood cells (Orth et al., 2005). 3) Topical application of supernatant from hypoxic alveolar macrophages produces mast cells degranulation and increased leukocyte-endothelial adherence in normoxic cremaster and mesentery post-capillary venules (Chao et al., 2009a; Gonzalez et al., 2007a). 5) Primary alveolar macrophage cultures undergo a respiratory burst within 15 min of reduction of PO_2 ; in contrast, neither peritoneal mast cells nor peritoneal macrophages primary cultures are stimulated by hypoxia within this time frame (Chao et al., 2009a). However mast cells are rapidly activated when immersed in supernatant of hypoxic alveolar macrophages, but not of hypoxic peritoneal macrophages (Chao et al., 2009a).

The nature of the mediator(s) released by alveolar macrophages to trigger the inflammation is not clear. Alveolar macrophages are a rich source of oxidants, cytokines, chemokines, growth factors, and arachidonic acid metabolites, which can be secreted in response to several stimuli, including hypoxia and ischemia/reperfusion (Chao et al., 2009a; Naidu et al., 2002; VanOtteren et al., 1995). Chemokines are primary products of activated alveolar macrophages (Ben-Baruch et al., 1995). The chemokine super family has been subdivided into four sub-families, which differ with respect to the number and arrangement of the conserved cysteine residues at the N terminus of the primary amino acid sequence (Murphy, 1996). Monocyte chemoattractant protein-1 (MCP-1/CCL2),

the prototype of the CC-chemokine subfamily, is an attractive candidate for a mediator of the systemic inflammation of alveolar hypoxia: MCP-1 is endowed with chemotactic and activating properties for macrophages (Fuentes et al., 1995), CD4+/CD8+ T lymphocytes (Carr et al., 1994) and mast cells, and is critically involved in the regulation of inflammatory processes. MCP-1 induces mast cells degranulation and leukocyte endothelial adherence in the rat cremaster microcirculation (Wan et al., 2003). A possible role of MCP-1 as a putative mediator of the systemic inflammation of alveolar hypoxia is suggested by our previous observation that primary cultures of alveolar macrophages, but not of peritoneal macrophages or mast cells, release MCP-1 within minutes of a reduction in PO_2 (Chao et al., 2009a). Release of MCP-1 by alveolar macrophages has also been observed after hypoxia-reoxygenation (Krishnadasan et al., 2003; McCourtie et al., 2008; Penner, 1988), which influences distal organ damage in hemorrhagic shock (Frink et al., 2007) and activates mast cells to elicit microvascular inflammation (Conti et al., 1995; Wan et al., 2003).

The objective of the present experiments was to provide definitive evidence concerning a possible role of MCP-1 as the circulating mediator that initiates the systemic inflammation of alveolar hypoxia. We reasoned that if MCP-1 does play this role, the plasma concentration of MCP-1 should increase in animals exposed to hypoxia, the time course and magnitude of this increase should be sufficient to elicit systemic effects, and these effects should be clearly ascribed to activation

of mast cells by MCP-1 released from alveolar macrophages. The results obtained show that MCP-1 is indeed a key initiator of the systemic inflammation of alveolar hypoxia.

4.2 Methods

The general methods and techniques utilized are described in detail in chapter 2.

4.2.1 Filtration experiments

Samples of normoxic plasma obtained from intact rats and from normoxic, alveolar macrophage-depleted rats, and samples of normoxic alveolar macrophages supernatant were filtered using Amicon Ultra filter device (30KDa, 50KDa or 100KDa) and centrifuged at 14000×g for 30 minutes. Usually, two 0.5 aliquots from each sample were filtered. The filtrates were collected and diluted to original volume with serum-free DMEM. The retained fractions were collected by centrifuging the filter upside down in a clean centrifuge tube at 1000×g for 2 minutes, then diluted to original volume with serum-free DMEM. In every case, 0.4×10^6 isolated mast cells were suspended in 0.4ml aliquots of filtrate or of the retained fraction. Four μ l of 3000ng/ml MCP-1 (dissolved in serum free DMEM) was added to each culture to yield final concentration of 30 ng/ml. The cells were incubated for 20 minutes and then centrifuge at 3000 rpm for 2 minutes. The

cells were resuspended in 40µl of ruthenium red (50 µg/ml) to yield cell concentration of 10^7 cell/ml. The percentage of degranulated mast cells was checked with cell smear using Nikon TE2000-S microscope. Photographs were obtained from 5 different areas of the slide, containing approximately 20 cells each area.

4.3 Results

4.3.1 Hypoxia-induced release of MCP-1 from isolated alveolar macrophages

Primary alveolar macrophage cultures were equilibrated with gas mixtures selected to reproduce the PO_2 to which alveolar macrophages may be exposed *in vivo* under various conditions. A gas mixture of 15% O_2 provides an environment similar to that of the normoxic alveolar gas, i.e. a PO_2 of approximately 100 Torr. The 10% and 5% O_2 gas mixtures provide alveolar PO_2 values observed in conditions of moderate hypoxia ($PO_2 \sim 70$ mm Hg) and severe hypoxia ($PO_2 \sim 35$ mm Hg). Equilibration with gas mixtures nominally containing 0% O_2 seldom results in total absence of O_2 in the solution; with the equilibration method used in these experiments PO_2 of the solution is approximately 5 mm Hg in these conditions (Chao et al., 2009a). While this is far below levels to which alveolar macrophages may be exposed *in vivo*, these values approximate the systemic microvascular PO_2 values that are observed in

rats breathing 10% O₂ (Dix et al., 2003; Shah et al., 2003) and to which mast cells and systemic tissue macrophages may be exposed during hypoxia.

The basal release of MCP-1 into the supernatant of primary cultures of alveolar macrophages equilibrated with 15% O₂ was 200 ±32 pg/10⁶ cells (N = number of cell cultures = 5). In contrast, MCP-1 released by alveolar macrophages during 30 minutes of equilibration with the hypoxic gas mixtures was nearly 20-fold greater (Table 2). However, there was no correlation between the magnitude of MCP-1 released and the oxygen tension of the equilibrating gas mixtures in the range of 0-10% O₂. Viability of alveolar macrophages, assessed by Trypan Blue exclusion at the end of the experiments ranged from 99.4±0.1% with 15% O₂ to 99.6±0.1% with 10% O₂.

Table 2 Hypoxia-induced release of MCP-1 from primary AMØ cultures

<i>Nominal % O₂</i>	<i>15%</i>	<i>10%</i>	<i>5%</i>	<i>0%</i>
N	5	5	5	5
Supernatant concentration of MCP-1 (pg/10 ⁶ cell)	200±32	4051±591*	3757±909*	4119±354*

Values are mean ± SE of 5 primary cultures of AMØ exposed to each of the gas mixtures

N = number of primary cultures exposed to each gas mixture

* P<0.01 vs. 15% O₂ group

4.3.2 CCR2b receptor antagonist prevents degranulation of isolated mast cells immersed in supernatant of hypoxic alveolar macrophages

Peritoneal mast cells were immersed in supernatant of alveolar macrophages exposed to normoxic (15% O₂) hypoxic alveolar macrophages (5% O₂) gas mixtures (Table 3). As expected (Chao et al., 2009a), mast cells immersed in normoxic alveolar macrophage supernatant did not degranulate, while hypoxic alveolar macrophage supernatant induced significant mast cell degranulation. On the other hand, peritoneal mast cells pretreated with the MCP-1 receptor antagonist RS-102895(10μM) showed no degranulation when immersed in supernatant of hypoxic alveolar macrophages. Pretreatment with RS-102895 did not affect C48/80-induced mast cells degranulation (Table 2). While RS-102895 blocks MCP-1 effects by binding to the specific CCR2b receptor (Futagami et al., 2008), C48/80, a basic mast cells secretagogue, induces mast cell degranulation via a receptor-bypassing action which includes activation of the Gi/o class of G proteins (Metcalf et al., 1997).

Table 3 The MCP-1 receptor antagonist, RS-102895, prevents degranulation of mast cells immersed in hypoxic AMØ supernatant

<i>% of degranulated mast cells</i>	<i>Supernatant of AMØ exposed to 15% O₂</i>	<i>Supernatant of AMØ exposed to 5% O₂</i>	<i>Supernatant of AMØ exposed to 5% O₂ + C48/80</i>
Untreated	3.6±0.5	74.7±0.3	87.5±1.7 ^s
RS-102895	-	14.2±1.3*	84.5±4.3 [#]

Mast cells from the same culture (0.4×10^6 cells) were immersed in supernatant of AMØ exposed to either 15 or 5% O₂. The supernatants contained RS-102895, the mast cell secretagogue c48/80, or both. Untreated AMØ supernatant contained neither of these. Mast cell

degranulation was assessed 20 min after immersion. N= number of cell cultures studied=3. * P<0.01 vs. corresponding untreated group; § P<0.01 vs. corresponding C48/80 group.

4.3.3 Plasma MCP-1 levels of intact and of alveolar macrophage-depleted conscious rats breathing 10% O₂

Hypoxia induced a significant increase of MCP-1 in plasma of rats with normal number of alveolar macrophages (Figure 6). The number of alveolar macrophages recovered by BAL in these rats, treated with PBS liposomes, was $7.74 \pm 0.34 \times 10^6$ cells/rat (N=5). MCP-1 was already increased at 5 minutes of hypoxia, and remained elevated at similar levels for the remainder of the hypoxic exposure. On the other hand, in the rats in which alveolar macrophages had been depleted with injection of clodronate-containing liposomes (number of alveolar macrophages recovered by BAL = $0.32 \pm 0.05 \times 10^6$ /rat; N=5) plasma MCP-1 concentration remained unchanged from normoxic values for the first 30 min, and increased significantly only after 60 min of hypoxia, at which time it reached a value of less than 50% of that seen in the rats with normal alveolar macrophage count.

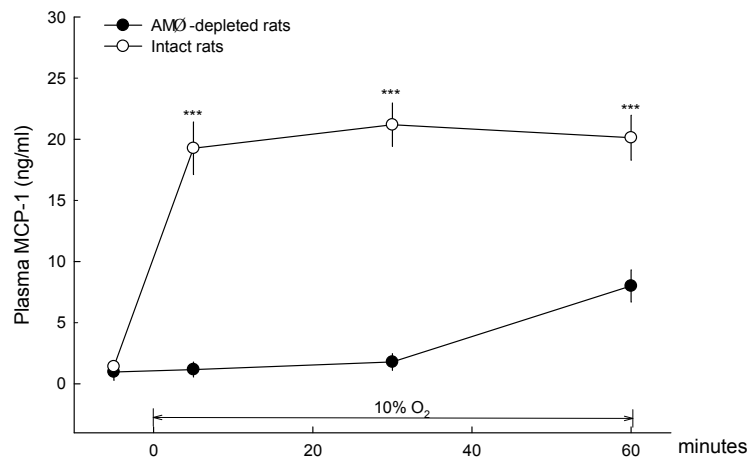


Figure 6

Plasma MCP-1 concentration in conscious AMØ- depleted (solid circles) and intact (open circles) rats before and during 1 h of breathing 10 % O₂. AMØ were depleted by tracheal instillation of clodronate-containing liposomes 4 d before the experiment. Intact rats received PBS-containing liposomes. Bars are mean ± SE, N= 5 rats per group in each data point: *** = P<0.01 vs. corresponding normoxic control: §= P <0.01 vs. corresponding value in intact rats.

Peritoneal mast cells (0.4×10^6 cells) obtained from normoxic rats were immersed in the plasma samples (0.4 ml) obtained from the conscious rats breathing 10% O₂. Essentially no degranulation was observed when peritoneal mast cells were immersed into the plasma samples obtained during the normoxic control period of either intact or alveolar macrophage-depleted rats (Figure 7). Mast cell degranulation increased significantly when the cells were immersed in plasma obtained when the intact rats were breathing 10% O₂; the percentage of degranulated mast cells was essentially the same in samples withdrawn at different times of hypoxia. In marked contrast, little degranulation occurred when

mast cells were immersed in plasma from the hypoxic, alveolar macrophage-depleted rats. This was the case even when mast cells were immersed in plasma from the 60 minute hypoxic sample, in which MCP-1 had increased above control levels.

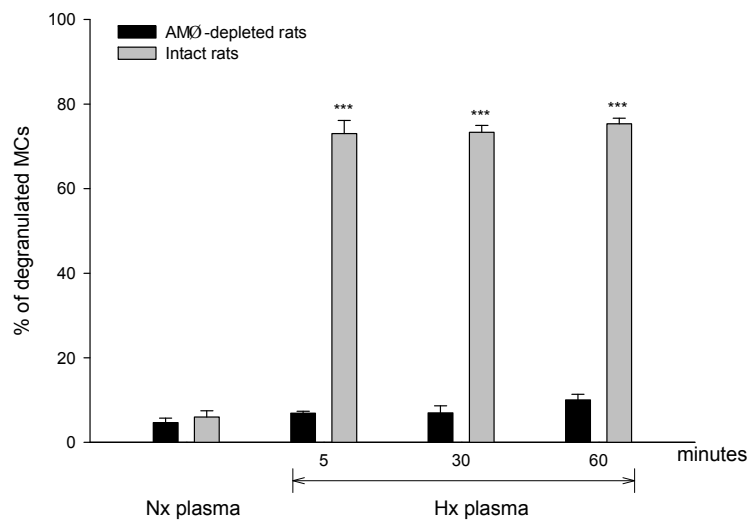


Figure 7

Percent of degranulated mast cells after immersion in rat plasma. Peritoneal mast cells (0.4×10^6 cells) from normoxic intact rats were immersed in 0.4 ml of plasma obtained from the rats depicted in Figure 6 at the times indicated in the horizontal axis. Black bars: AMØ-depleted rats; gray bars: intact rats. *** = $P < 0.01$ vs. corresponding normoxic control. § = $P < 0.01$ vs. corresponding value in intact rats

4.3.4 MCP-1 concentration-dependence of peritoneal mast cell degranulation

The data of the previous section show that, in general, degranulation occurred when mast cells were immersed in plasma from hypoxic intact rats, which had high MCP-1 levels, and did not degranulate when immersed in plasma of hypoxic alveolar macrophage-depleted rats, which showed lower levels of MCP-1. To further explore the dependence of mast cell degranulation on MCP-1 concentration, mast cells were exposed to increased concentrations of MCP-1 dissolved in plasma obtained from normoxic rats, in supernatant of normoxic alveolar macrophages, and in normoxic, serum-free DMEM. In all cases 0.4×10^6 mast cells were immersed in 0.4 ml of solution. Mast cell degranulation was observed 20 min after immersion. Figure 8 plots percent of degranulated mast cells as a function of MCP-1 added to the solution. The data obtained when mast cells were immersed in the plasma obtained during 10% O₂ breathing –from both intact and alveolar macrophage-depleted rats (Figure 6) - are included for comparison. For these samples, the percent of mast cell degranulation observed was plotted as a function of the plasma MCP-1 concentration determined by ELISA.

Mast cells immersed in normoxic rat plasma obtained or in normoxic alveolar macrophage supernatant showed essentially the same MCP-1 concentration-dependent mast cell degranulation. This relationship also fits the data obtained in mast cells immersed in plasma withdrawn from intact rats breathing 10% O₂ (Figure 8, solid red diamonds). Mast cells immersed in the plasma samples obtained when the alveolar macrophage-depleted rats were breathing 10% O₂

showed no increase in degranulation (Figure 8, open red diamonds). This was the case even for the sample obtained at 60 min of hypoxia which showed MCP-1 concentration of 8 ± 1.3 ng/ml. This concentration would produce degranulation of approximately 40% of the mast cells immersed in normoxic plasma or alveolar macrophage supernatant (Figure 8). This suggests that alveolar macrophages are necessary for plasma to elicit MCP-1-mediated mast cell degranulation.

In marked contrast to normoxic plasma and normoxic alveolar macrophage supernatant, MCP-1 added to normoxic culture medium failed to increase mast cell degranulation, even at the highest MCP-1 concentration. A possible explanation for this is that a substance(s) present in normoxic plasma and in the supernatant of normoxic alveolar macrophages, but absent in plasma of alveolar macrophage-depleted rats, is necessary for MCP-1 to induce degranulation of mast cells *in vitro*.

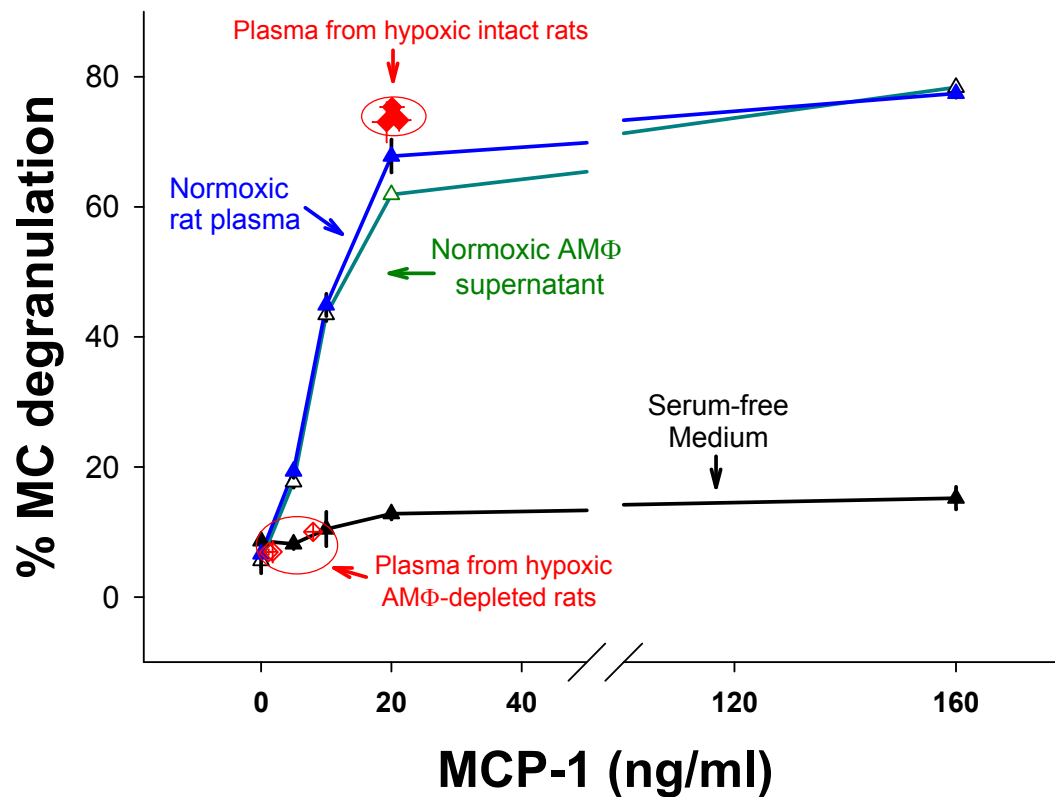


Figure 8

Peritoneal mast cells obtained from normoxic intact rats (0.4×10^6 cells) were immersed in 0.4 ml of solutions containing increasing concentrations of MCP-1. Mast cells were immersed in normoxic AMØ supernatant (green), in normoxic plasma (blue) and in serum-free DMEM culture medium (black). Data are mean \pm SE of 3 experiments per group in each data point. The red diamonds represent the data obtained immersing mast cells in the plasma drawn from the AMØ-depleted rats (open diamonds) and the intact rats (solid diamonds) breathing 10% O₂ depicted in Figure 7.

4.3.5 Filtration of plasma and alveolar macrophage supernatant

To obtain preliminary evidence of a putative co-factor of MCP-1, normoxic plasma from intact and from alveolar macrophage-depleted rats, as well as

supernatant of normoxic alveolar macrophages, were filtered through pores of different sizes. Aliquots of MCP-1 were added to the filtrate as well as the retained fractions to obtain a concentration ~ 30 ng/ml. This concentration produces degranulation of $\sim 75\%$ of mast cells immersed in plasma of intact rats or in alveolar macrophage supernatant (Figure 7). Peritoneal mast cells obtained from normoxic intact rats were then added to the fractions containing MCP-1, and mast cell degranulation was determined 20 min later. The results obtained using either plasma from intact rats or alveolar macrophage supernatant were essentially (Table 4). On the other hand, no degranulation occurred when mast cells were added to any of the filtrate or of the retained fraction of plasma obtained from alveolar macrophage-depleted rats.

MCP-1 induced mast cell degranulation in only two cases: in the fraction of plasma or alveolar macrophage supernatant retained by the 30 KDa filter; and in the filtrate of the 100 KDa filter. No mast cell degranulation was observed in either fraction when the 50KDa filter was used. The best explanation for these results is that the MCP-1 cofactor is composed of two elements: one with a size between 30 and 50 KDa, and another with a size larger than 50 and smaller than 100 KDa. Both elements would be together in the retained fraction of the 30 KDa filter, as well as in the filtrate of the 100 KDa filter. Mast cell degranulation in either fraction of the 50 KDa filter would not occur because the co-factor components are separated by the filter.

Table 4 Effect of different filtered fractions of normoxic plasma and AMØ supernatant on degranulation of immersed mast cells

Pore size	Fraction	% of degranulated mast cells		
		Plasma of intact rats (n=3)	Supernatant of normoxic AMØ (n=3)	Plasma of AMØ-depleted rats (n=3)
30KDa	retained	76.7±1.3*	72.6±1.6*	5.4±1.1
	filtrate	5.2±1.3	4.8±1.5	6.2±1.3
50KDa	retained	5.4±2.6	4.3±0.9	6.0±1.4
	filtrate	5.1±0.4	5.6±1.3	6.7±0.2
100KDa	retained	3.7±1.5*	5.4±1.0*	6.8±0.8
	filtrate	75.0±3.4	75.2±1.2	6.1±1.0

MCP-1 (30 ng/ml) and mast cells (0.4×10^6 cells in a final volume of 0.4 ml) were added to the retained fraction or the filtrate. Mast cell degranulation was assessed 20 min after immersion. N = number of primary cultures. * $P < 0.01$ vs. corresponding filtrate

In addition to providing evidence of the existence of this co-factor, the data also suggest that the co-factor originates in alveolar macrophages: similar results were obtained immersing mast cells into plasma or in alveolar macrophage supernatant suggesting that this substance is released by alveolar macrophages into the plasma; this is supported by the finding that there is no evidence of the presence of a co-factor in plasma of alveolar macrophage-depleted rats. These data are consistent with the observation that mast cells did not degranulate when immersed in the plasma sample obtained after 60 min of hypoxia in the alveolar macrophage-depleted rats, which showed a significant increase in MCP-1 concentration (Figure 8).

4.3.6 CCR2b receptor antagonist prevents the inflammatory response to alveolar hypoxia in the mesentery of intact rats

These experiments were carried out to explore the possible role of MCP-1 in the systemic inflammation of alveolar hypoxia in anesthetized intact rats. Figure 9 shows representative microphotographs of a mesenteric post-capillary venule of an intact rat obtained before (left) and at 30 min of 10% O₂ breathing (center). The microphotograph at the right shows the effect of 30 min of alveolar hypoxia in a rat pretreated with RS-102895 (10 µM applied topically). Hypoxia in the untreated rats resulted in the expected inflammatory response: increases in mast cells degranulation index and in leukocyte-endothelial adherence. These effects of alveolar hypoxia were absent in the rats pretreated with RS-102895.

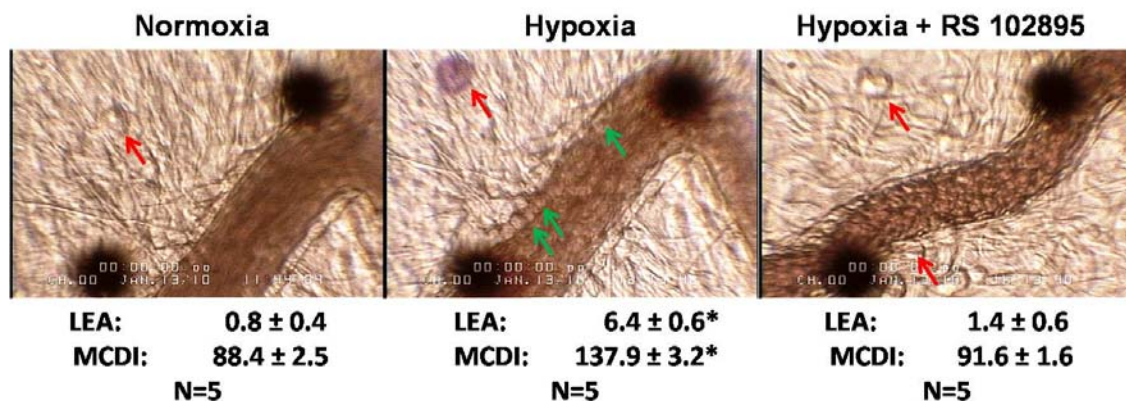


Figure 9

Representative microphotographs of post capillary mesenteric venules of intact rats. The large black dots are used to align the optical Doppler velocimeter used to measure red cell velocity, and occasionally are moved from the vessel center to obtain a better view of the leukocyte-

endothelial interface for photographs. The left and center photographs were taken before and at 30 min of breathing 10% O₂, respectively. The red arrows point to mast cell and the green arrows point to adherent leukocytes. Hypoxia induces mast cell degranulation, as shown by the uptake of ruthenium red, and increased adherence of leukocytes to the endothelium. The photograph on the right shows a venule of an intact rat pretreated with the CCR2b receptor antagonist RS-102895 (10 µM applied topically). The photograph was obtained at 30 min of 10% O₂ breathing. In contrast with the photograph in the center, pretreatment with RS-102895 prevents mast cell degranulation and leukocyte-endothelial adherence of alveolar hypoxia. The numbers below the photographs represent mean ± SE of 5 rats in each group. LEA: leukocyte-endothelial adherence as determined by leukocytes /100 µm. MCDI: Mast cell degranulation intensity, arbitrary units.

4.3.7 Effects of topical MCP-1 on the microcirculation of intact and alveolar macrophage-depleted rats

The data of Figure 8 suggest that MCP-1 participates in the mesenteric mast cells degranulation and increased leukocyte-endothelial adherence that occur at onset of alveolar hypoxia. Further evidence on this was obtained using an alternative approach, namely determining the effect of administration of MCP-1 to normoxic rats with normal alveolar macrophage count.

Topical application of MCP-1 (30 ng /ml dissolved in serum-free DMEM) to the mesentery of intact, normoxic rats mimicked the response to alveolar hypoxia (compare left and center panels of Figure 9). Pretreatment of the mesentery with RS-102895 prevented the inflammatory response (Figure 10, right).

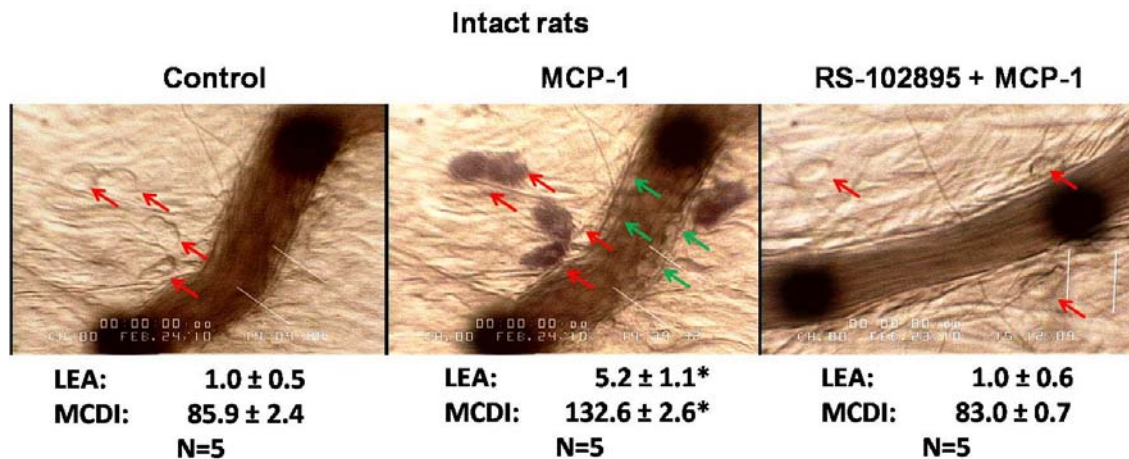


Figure 10

Mesenteric post capillary venule immediately before (left) and 30 min after topical application of MCP-1 (30 ng/ml dissolved in serum-free DMEM). Red arrows: mast cells, green arrows: adherent leukocytes. Right panel: mesenteric venule 30 min after application of MCP-1 in a rat topically pretreated with RS-102895 (10 μ M), a CCR2b antagonist. Data are mean \pm SE of 5 rats per group. * $P < 0.01$ vs. corresponding control

In contrast, the effect MCP-1 on the mesentery of alveolar macrophage-depleted rats depended on the vehicle into which MCP-1 was dissolved. MCP-1, 30 ng/ml dissolved in serum-free DMEM, had no effect on the mesentery of alveolar macrophage-depleted rats (Figure 11, left). On the other hand, topical application of equal amounts of MCP-1 dissolved in plasma obtained from normoxic rats with normal alveolar macrophage count produced an inflammatory response (Figure 11, right). The effects of MCP-1 here were similar to those seen when MCP-1 dissolved in serum-free DMEM was applied to intact rats (Figure 10, center).

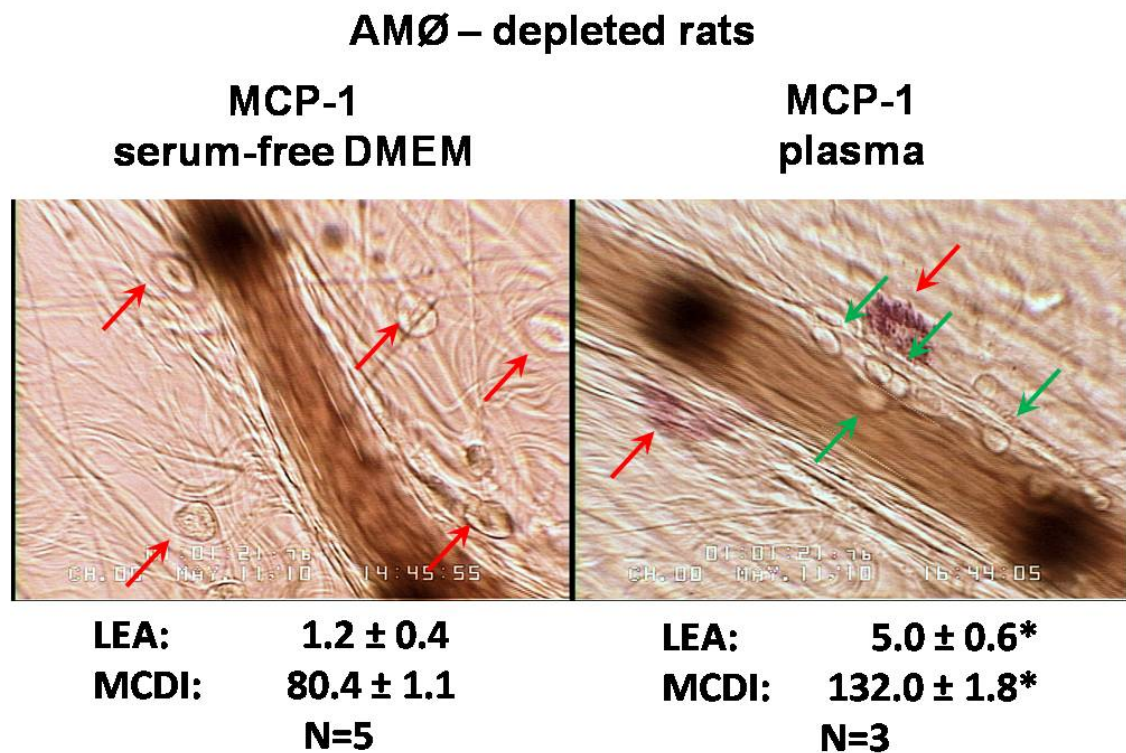


Figure 11

Left: Post capillary mesenteric venule of an AMØ-depleted rat 30 min after topical application of 30 ng/ml of MCP-1 dissolved in serum-free DMEM. Right: Post capillary mesenteric venule of an AMØ-depleted rat 30 min after topical application of 30 ng/ml of MCP-1 dissolved in plasma from intact rats. Data are mean ± SE of 5 rats in each group. * P<0.01 vs. corresponding control

4.4 Discussion

The findings of the present experiments demonstrate that MCP-1 plays a central role in the initiation of the systemic inflammatory response to alveolar hypoxia. The results also show that a co-factor, normally present in normoxic plasma and originated in alveolar macrophages, is necessary for the stimulatory effect of MCP-1 on mast cells.

Our previous work has provided compelling evidence that the systemic inflammation of hypoxia is not initiated by the reduced tissue PO_2 , but rather by a mediator released from alveolar macrophages and transported by the circulation (Chao et al., 2009a; Dix et al., 2003; Gonzalez et al., 2007a; Gonzalez et al., 2007b; Orth et al., 2005; Shah et al., 2003). A role for alveolar macrophage-borne MCP-1 as the putative mediator was suggested by the previous finding that primary cultures of alveolar macrophages, but not of peritoneal mast cells or peritoneal macrophages, release MCP-1 within 30 min of exposure to hypoxia (Chao et al., 2009a). Of 12 different chemokines and cytokines investigated, MCP-1 was the only mast cell secretagogue released by alveolar macrophages within a time frame compatible with a role as a trigger of the inflammation. Hypoxia-induced release by alveolar macrophages of a mast cell secretagogue such as MCP-1 is consistent with the observation that cultures of hypoxic alveolar macrophages, but not of hypoxic peritoneal macrophages, elicited degranulation of immersed mast cells and induced inflammation in tissues of normoxic animals (Chao et al., 2009a; Chao et al., 2009b; Gonzalez et al., 2007a). While these data provide evidence of the participation of MCP-1 in the inflammatory process, several criteria must be met to demonstrate that MCP-1 is the alveolar macrophage-borne circulating agent that initiates the systemic inflammatory cascade: First, reduction of alveolar PO_2 in intact animals must result in an increase in plasma MCP-1 concentration, and this increase must follow a pattern consistent with the time course of the inflammatory response.

Second, alveolar macrophages must be identified as the source of the elevated plasma MCP-1 levels in intact animals. Third, the plasma concentration of MCP-1 observed during hypoxia must be sufficient to elicit activation of mast cells, the target of the putative circulating mediator. Finally, evidence must be provided showing that MCP-1 is directly involved in the systemic inflammatory response to alveolar hypoxia in intact animals. The data presented here show that all these criteria were met to identify MCP-1 as the agent that initiates the systemic inflammation of hypoxia. In addition, the results indicate that a co-factor present in normoxic biological fluids is necessary for MCP-1 to activate mast cells. The evidence supporting a role for MCP-1 as the key initiator of the inflammation will be discussed first, followed by discussion of the data that point to the existence of a co-factor for MCP-1.

4.4.1 Alveolar macrophage-borne MCP-1 initiates the systemic inflammation of alveolar hypoxia

Breathing 10% O₂ elicited a rapid and sustained increase in plasma MCP-1 concentration in intact, conscious rats (Figure 6). Within 5 minutes, MCP-1 had reached the levels that were maintained for 1 h of hypoxia. This rapid increase matches the time course of the mast cell degranulation and leukocyte-endothelial adherence observed in the mesenteric and cremaster microcirculations of rats exposed to alveolar hypoxia (Shah et al., 2003; Steiner et al., 2003; Wood et al., 1999b).

The major source of the initial increase in plasma MCP-1 is evidently alveolar macrophages: First, reduction of PO₂ does induce a rapid release of MCP-1 from primary cultures of alveolar macrophages (Table 2). The effectiveness of alveolar macrophage-borne MCP-1 in inducing mast cell degranulation is supported by the inhibitory effect of RS-102985 on degranulation of mast cells immersed in hypoxic alveolar macrophage supernatant (Table 3). Second, depletion of alveolar macrophages by administration of clodronate liposomes prevented the increase in plasma MCP-1 observed during the first 30 min of hypoxia in conscious rats. At 60 min of hypoxia, plasma MCP-1 in the alveolar macrophage-depleted rats had increased to less than 50% of the value observed in the corresponding sample in the intact animals (Figure 6). It is not likely that the small number of alveolar macrophages remaining after 4 days of clodronate liposome treatment -less than 5% of the number recovered by BAL from the rats treated with PBS liposomes- is responsible for this increase. Besides the disproportionate levels of plasma MCP-1 in relation to the number of alveolar macrophages, it would be expected that MCP-1 released from these remaining alveolar macrophages would follow a time course similar to that observed in the control rats. Cells other than alveolar macrophages are probably the main contributors to the late increase in plasma MCP-1 in this case (Sharma et al., 2007). Whether the stimulus for the delayed release of MCP-1 is the reduced tissue PO₂ or some other factor associated with hypoxia in the intact animal is not known. Nevertheless, while MCP-1 from these alternative sources may

contribute to the systemic inflammation at later times, the time course indicates that it does not play a role in the rapid onset of the inflammation. Accordingly, these results indicate that alveolar macrophages are the predominant source of the early increase in plasma MCP-1 during hypoxia.

A second criterion that must be met by the putative circulating mediator of the inflammation is that its plasma concentration during hypoxia must be sufficient to activate mast cells. Immersion of mast cells in the plasma samples drawn from the intact conscious rats breathing 10% O₂, in which an elevated MCP-1 level was documented (Figure 6), produced substantial degranulation (Figure 7). No degranulation was observed when mast cells were immersed in normoxic plasma of intact or of alveolar macrophage-depleted rats, or in the plasma of the hypoxic, alveolar macrophage-depleted rats (Figure 7). Addition of MCP-1 to normoxic plasma or normoxic alveolar macrophage supernatant produced concentration-dependent degranulation of immersed mast cells (Figure 8). The percent of degranulated mast cells immersed in the plasma samples drawn from the intact rats breathing 10% O₂ (Figure 7) was close to that seen when mast cells were immersed in normoxic plasma or normoxic alveolar macrophage supernatant with similar MCP-1 concentrations (Figure 8). These results indicate that the plasma MCP-1 level reached by the hypoxic rats with normal alveolar macrophage count is sufficient to induce mast cell degranulation, suggest that mast cell degranulation occurred as a result of the elevated MCP-1 concentration, and was not the result of other factors associated with hypoxia.

The participation of MCP-1 in the inflammation induced by alveolar hypoxia in intact animals was investigated using complementary approaches. On one hand, pretreatment of the mesentery with RS-102985 prevented the rapid degranulation of perivascular mast cells and the increased leukocyte-endothelial adherence to mesenteric venules that is observed in the intact rats breathing 10% O₂ (Figure 9). RS-102895 is a selective antagonist of CCR2b, the putative MCP-1 receptor expressed in mast cells (Ko et al., 2006) and in endothelial cell membranes (Schols et al., 1994). Secondly, topical application of MCP-1 onto the mesenteric microcirculation of normoxic intact rats reproduced the inflammatory response to systemic hypoxia (Figure 10). This response was prevented by administration of RS-102985 in the same dose used to block the effect of alveolar hypoxia (Figure 9). Both RS-102985 and MCP-1 were administered topically. This avoided possible systemic responses to these agents which could influence the results. The MCP-1 concentration of the solutions applied approximated the plasma MCP-1 concentration observed in intact rats during hypoxia.

In summary, a combination of *in vivo* and *in vitro* data show that alveolar macrophages release MCP-1 under hypoxia, and that this release leads to an increase in plasma concentration of MCP-1 with a time course compatible with that of the systemic inflammation. Furthermore, the plasma MCP-1 level attained at the onset of hypoxia is sufficient to induce mast cell degranulation, the first step in the systemic inflammatory cascade. Finally, participation of MCP-1 in the

systemic inflammation of alveolar hypoxia is supported by the attenuation of the inflammation by MCP-1 receptor blockade in rats breathing 10% O₂. This was complemented by the observation that administration of MCP-1 to normoxic rats replicates the mesenteric inflammation produced by hypoxia.

4.4.2 Evidence supporting the existence of a MCP-1 co-factor

While these results provide substantial evidence supporting MCP-1 as the alveolar macrophage-borne circulating mediator which initiates the inflammation of alveolar hypoxia, it is also apparent that MCP-1 requires a co-factor to activate mast cells at concentrations observed under physiologically relevant conditions.

This was initially suggested by the observation that MCP-1 did not produce degranulation of mast cells immersed in normoxic cell culture medium, even at concentrations that were effective in plasma or in alveolar macrophage supernatant (Figure 8). This finding is consistent with earlier reports of a lack of effect of MCP-1 on degranulation of *in vitro* mast cell cultures (Alam et al., 1992; Fureder et al., 1995). In fact, release of histamine by rat peritoneal mast cell cultures was observed only when MCP-1 was administered in concentrations 10 to 20-fold greater than those seen in plasma of hypoxic rats; lower concentrations had no effect (Conti et al., 1995). These results are in marked contrast with the documented effectiveness of MCP-1 in activating mast cells *in vivo* (Wan et al., 2003).

The filtration experiments provided more direct evidence for the existence of such a co-factor in normoxic plasma and alveolar macrophage supernatant. Mast cell degranulation induced by MCP-1 was interpreted as an indicator of the presence of the co-factor in a given fluid fraction. The results, which were essentially the same in plasma from intact normoxic rats and in normoxic alveolar macrophage supernatant, suggest that the co-factor is composed of at least two fractions: one with a molecular weight between 30 and 50 kDa and the other with a molecular weight between 50 and 100 kDa (Table 4).

Several lines of evidence suggest that the co-factor is constitutively secreted by alveolar macrophages and is normally present in plasma and extracellular fluid. First, alveolar macrophages are necessary for MCP-1 to induce mast cell degranulation: mast cells immersed in plasma obtained from alveolar macrophage-depleted rats after 60 min of 10% O₂ breathing did not undergo degranulation. This was in spite of the fact that this sample exhibited a MCP-1 concentration that produces degranulation of 40-50% of mast cells immersed in plasma from intact rats or in alveolar macrophage supernatant (Figure 8). These results are consistent with the results of the filtration experiments, which showed no MCP-1-induced mast cell degranulation in any of the fractions of plasma from alveolar macrophage-depleted rats. Further support for the idea of an alveolar macrophage-borne co-factor for MCP-1 was provided by the results depicted in Figures 10 and 11. Topical MCP-1 dissolved in serum-free DMEM replicated the response to hypoxia in the mesentery of intact, normoxic rats (Figure 10), while it

had no effect in the alveolar macrophage-depleted rats (Figure 11). On the other hand, MCP-1 dissolved in plasma of rats with normal alveolar macrophage count did produce an inflammatory response in the alveolar macrophage-depleted rats. These results imply that absence of the co-factor in the mesenteric environment of the alveolar macrophage-depleted rats prevented the mast cell degranulation when MCP-1 was dissolved in DMEM; however, when the co-factor was provided using plasma of intact rats as a vehicle, MCP-1 did elicit mast cell degranulation in the mesentery of alveolar macrophage-depleted rats.

These results provide persuasive evidence of an alveolar macrophage-borne co-factor of MCP-1; its nature, however, remains unknown. Since this substance appears to be a normal component of body fluids which is not expressed exclusively under conditions of hypoxia, efforts directed to its identification are likely to be complex and extensive, and as such fall outside the scope of the present studies. The central question addressed by this research is whether MCP-1 is the alveolar macrophage-borne mediator of the systemic inflammation of hypoxia. The data strongly support this hypothesis. Within this context, the results indicate that a co-factor normally present in biological fluids is necessary for physiologically relevant concentrations of MCP-1 to activate mast cells. This issue should be considered when analyzing *in vitro* experiments involving MCP-1, when this co-factor may not be present.

4.4.3 Functional relevance of these findings

The present and previous studies (Chao et al., 2009a; Gonzalez et al., 2007a) highlight the systemic effects of alveolar macrophage activation. In contrast with their better known intrapulmonary effects, the extrapulmonary consequences of activation of alveolar macrophages have attracted less attention. Recently, evidence has been accumulating pointing to a systemic inflammatory effect of activation of alveolar macrophages secondary to phagocytosis of inhaled particulate matter. Inflammatory mediators released by alveolar macrophages enter the circulation to produce inflammation and endothelial dysfunction in systemic microvascular beds (Ishii et al., 2005; Nurkiewicz et al., 2006; Simkhovich et al., 2008; Tamagawa et al., 2008; van Eeden et al., 2001). It has been proposed that these alveolar macrophage-mediated responses underlie the elevated cardiovascular morbidity associated with environmental pollution (Simkhovich et al., 2008). The phenomenon described in this research represents an example of systemic response to alveolar macrophages activated by another stimulus, in this case reduced alveolar PO₂.

Clinical conditions as well as experimental interventions associated with alveolar hypoxia are accompanied by systemic effects, and in several of these, a systemic inflammatory component has been identified. Examples are the sarcopenia and cardiovascular abnormalities of chronic obstructive pulmonary disease (Agusti and Soriano, 2008), the insufficient hemopoietic response in pulmonary fibrosis (Tsantes et al., 2003b), and the cardiovascular and metabolic

dysfunctions in sleep apnea (Jelic et al., 2008). In acute conditions, systemic responses with a possible inflammatory link include the multiple organ failure secondary to atelectasis (Kisala et al., 1993), acute lung injury (Puneet et al., 2005; St John et al., 1993) and pulmonary contusion (Perl et al., 2005), the systemic inflammation of pneumonia (Fernandez-Serrano et al., 2003) and the acute illnesses of high altitude (Basnyat and Murdoch, 2003). It is possible that inflammation does not play a causal role in every one of these examples; however, it is likely that inflammation modifies their development and outcome. The phenomenon described here could play a role in the initiation of the systemic inflammation observed in some of these conditions. In this respect, our studies in intact animals entailed a fairly severe degree of hypoxia: alveolar PO_2 values in rats breathing 10% O_2 typically decrease to 45-50 Torr. This PO_2 is observed, for instance, at an altitude of ~ 5,000m, or in clinical conditions in which pulmonary function is seriously compromised. However, alveolar macrophage cultures released MCP-1 when exposed to a PO_2 of ~ 70 Torr and the response to this less severe decrease in PO_2 was the same as that observed at lower PO_2 values (Table 2). This would suggest that clinical conditions presenting relatively moderate reductions in alveolar PO_2 may elicit alveolar macrophage-initiated systemic inflammation

It has been demonstrated that alveolar hypoxia also produces an inflammatory response in the lungs, and that alveolar macrophages play an important role in this response. Rats breathing 10% O_2 show extravasation of albumin and

expression of inflammatory cytokines (Beck-Schimmer et al., 2001; Madjdpour et al., 2003). These changes are attenuated by the selective depletion of alveolar macrophages. In addition, hypoxia induces the expression of neurokinin-1 receptors in the lung, which in turn lead to inflammatory responses mediated by IL-1, IL-6 and TNF- α (Zee et al., 2006). Whether similar mechanisms underlie the inflammatory responses of the lung and the systemic circulation to reduced alveolar PO₂ should be the subject of future research

Finally, It should be acknowledged, that exposure of an intact organism to hypoxia is a complex stimulus which elicits multiple responses. Mechanisms with a slower time course, in particular the changes in gene expression initiated by the reduction in PO₂ are likely to modify the effects of this initial response at later times in the course of hypoxia.

In summary, the present studies provide substantial evidence in support of the idea that the systemic inflammatory response to a reduction in alveolar PO₂ is not initiated by the reduction in PO₂ in the systemic microcirculation, but rather by activation of mast cells by MCP-1 released by alveolar macrophages and transported by the circulation.

CHAPTER 5

Role of renin-angiotensin system in the inflammatory response induced by activated mast cells

5.1 Introduction

Alveolar hypoxia, induced by 10% O₂ breathing, produced an initial arteriolar vasoconstriction in skeletal muscle which was followed by vasodilation. Plasma from hypoxic rats produced not only increased venular leukocyte-endothelial interactions and mast cell activation, but also produced local arteriolar vasoconstriction in the normoxic cremaster; both the inflammation and the arteriolar vasoconstriction were attenuated by non-selective Ang II receptor antagonists and ACE inhibitors (Orth et al., 2005). These agents also prevented the inflammation induced by alveolar hypoxia in intact rats (Gonzalez et al., 2007b). These results suggest a participation of the RAS in the inflammatory cascade initiated by alveolar hypoxia. Ang II is a potent vasoconstrictor implicated in inflammatory processes in the cardiovascular system. In addition to the circulating RAS, local tissue RAS plays an important role in several pathophysiological processes, including cardiovascular inflammation (Paul et al., 2006). Ang II can cause increased leukocyte-endothelial interactions and leukocyte recruitment, increased vascular permeability, and eventually tissue remodeling (Duprez, 2006; Ferrario and Strawn, 2006). Similar to systemic

hypoxia, Ang II stimulates leukocyte-endothelial interactions in postcapillary venules via generation of reactive oxygen species (ROS) (Suzuki et al., 2006); these effects can be blocked by antioxidant administration (Wood et al., 1999a). While Ang II induces inflammation in the normoxic mesentery, it does not elicit mast cell degranulation and its effects are not blocked by cromolyn (Piqueras et al., 2000). On the other hand, the inflammatory effects of stimulation of mast cells with C48/80, a mast cell secretagogue, are attenuated by ACE inhibition and by Ang II receptor blockade in cremaster (Gonzalez et al., 2007b). The inflammation was not due to increased levels of Ang II or of renin in the donor plasma, suggesting that plasma from hypoxic rats activated the tissue RAS, which occurred downstream of mast cell degranulation.

While it is clear that the RAS is activated by mast cells, the underlying mechanism is uncertain. Two questions need to be answered: the mechanism of formation of Ang II and the origin of renin. There are two possible Ang II formation mechanisms by which mast cells may activate the tissue RAS: chymase- Ang II forming pathway and ACE- Ang II forming pathway (Akasu et al., 1998). The presence of chymase, an angiotensin converting enzyme (Batlle et al., 2006; Miyazaki et al., 2006), is species-dependent. A mast cell chymase was identified as the major Ang II-forming pathway in the human heart (Urata et al., 1990). In rodents, Ang II formation is primarily ACE-related, but chymase immunoreactivity is also detected in mast cells (Huntley et al., 1990). The biochemical and physiological roles of chymase seem to vary by subtypes (α and

β -chymases) (Sanker et al., 1997). For example, rat chymase I, which was identified in rat peritoneal mast cells, belongs to β -chymases subtype and is an Ang II-degrading enzyme rather than an Ang II-forming enzyme (Chandrasekharan et al., 1996; Le Trong et al., 1987; Sanker et al., 1997). However, rat chymase-3, which belong to α -chymases subtype, converts Ang I to Ang II by cleaving the Phe⁸-His⁹ bond in Ang I (Balcells et al., 1997). Non-ACE-dependent Ang II formation is also found in rat heart (Balcells et al., 1997; Muller et al., 1998), vasculature (Ideishi et al., 1990; Juul et al., 1987; Leite et al., 1997), cultured vascular cells, such as endothelial and smooth muscle cells (Andre et al., 1990; Ideishi et al., 1993). Previous data from our laboratory suggest that hypoxia produced similar inflammatory effects in both mesentery and cremaster, including mast cell degranulation, leukocyte-endothelial adhesive interaction. In cremaster microcirculation experiments, we observed that topical application of Ang II, C48/80 or hypoxic alveolar macrophage supernatant produced similar increases in leukocyte-endothelial adhesive interaction (Gonzalez et al., 2007a). Ang II receptor blockade and ACE inhibition had essentially the same effects on inflammation induced by either alveolar hypoxia or plasma from hypoxic rats (Gonzalez et al., 2007b). Since the ACE inhibitors traditionally used do not act on mast cell chymase (Fildes et al., 2005; Miyazaki et al., 2006; Takai and Miyazaki, 2002), these results suggest that conversion from Ang I to Ang II on cremaster was entirely due to ACE and not to chymase (Gonzalez et al., 2007b).

With respect to the mechanism of activation of the RAS, renin is the enzyme that catalyzes the first and rate-limiting step of RAS. Rat cardiac mast cells contain renin (Silver et al., 2004) and can release renin to activate RAS under conditions of ischemia-reperfusion (Mackins et al., 2006). Whether rat peritoneal mast cells contain renin is unknown.

The aims of the present experiments were two: first, to determine if, as seen in skeletal muscle, the RAS is involved in the mesenteric inflammation of hypoxia. Similar patterns of inflammation occurring in different vascular beds would be consistent with our general hypothesis of an inflammatory response initiated by a circulating mediator released by alveolar macrophages. Secondly, we wanted to determine if the activation of the RAS by mast cells involves the participation of mast cell renin.

5.2 Methods

The general methods and techniques utilized are described in detail in chapter 2.

5.2.1 Immunocytochemistry

Mast cells were resuspended in DMEM to yield cell concentration of 1.25×10^4 cells/100 μ l. A slide was placed into the slide holder with a slide filter on top. The slide funnel was placed onto the slide filter, lining up the holes to make sure that the cells would be forced onto the slide. 200 μ l of the cell suspension containing a total of 2.5×10^4 cells was added to the slide funnel. The lid of the Cytospin was

carefully placed over the samples. The slide was spun at 1000 rpm speed for 10 minutes.

The slide was placed in methanol at 4°C for 15 minutes, following in 50% methanol at room temperature for 10 minutes and PBS at room temperature for another 10 minutes. After 3 washes with PBS, the cells were permeabilized for 30 minutes at room temperature with a solution containing 0.5% Triton X-100 dissolved in PBS. After 3 washes with 0.5% Triton X-100 dissolved in PBS, cells were quenched with 100 mM ammonium chloride for 15 minutes, following with 3% H₂O₂ in methanol for 30 minutes. After 3 washes with 0.5% bovine serum albumin (BSA) / 1% normal goat serum (NGS) in PBS, the slide was blocked by using Avidin/Biotin Blocking kit, and then by 1% BSA / 5% NGS in PBS at room temperature for 2 hours. Next, the slide was exposed to renin antibody (1:200) in 0.1% BSA / 2% NGS in PBS at 4°C overnight. Following 3 washes with 0.1% BSA in PBS, the slide was exposed to second antibody in 0.1% BSA / 2% NGS in PBS for 2 hours at room temperature. After 3 washes with 0.1% BSA in PBS, the slide was staining with DAB kit. After washing with water, the slide was mounted with PBS kept in 4°C. The slide was examined with a Nikon TE-2000 S microscope.

5.3 Results

5.3.1 Role of the RAS on the response of the microcirculation to hypoxic alveolar macrophage supernatant

Ang II was selected because of the known participation of RAS (Gonzalez et al., 2007b) and mast cells activation (Dix et al., 2003; Gonzalez et al., 2007b; McDonald and Wood, 2003; Steiner et al., 2003) in inflammation induced by alveolar hypoxia. Ang II and C48/80 produced similar inflammatory response in cremaster (Gonzalez et al., 2007a). Here, we show that topical application of Ang II (10 nM) produced an increase in leukocyte-endothelial adherence without producing mast cell degranulation (Figure 12A, N=5). This is consistent with our observation in cremaster, which indicates that same mechanism may be involved in the widespread inflammation in different microvascular beds.

Application of supernatant of alveolar macrophages equilibrated with 10% O₂ to the mesentery of rats pretreated with the non-specific Ang II receptor antagonist [Sar1,Thr8] Ang II (30 µg/kg/ min , continuously infused i.v. ,Figure 12B, N=5) or with the NADPH oxidase inhibitor apocynin (1 mM applied topically, Figure 12C, N=5) essentially produced the same results: although mast cell degranulation occurred in response to the application of supernatant, no increase in leukocyte-endothelial adherence was observed.

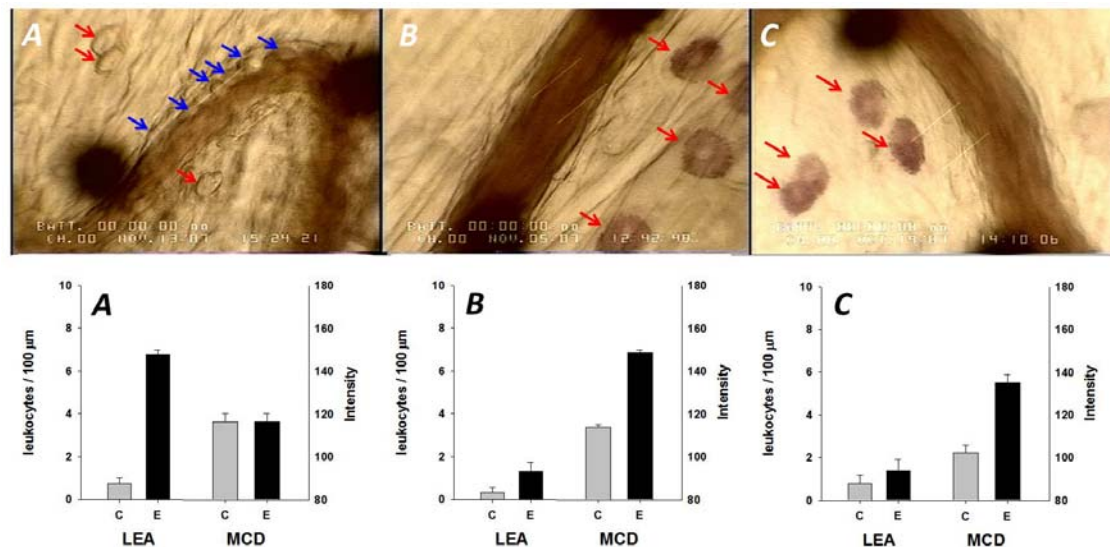


Figure 12 Role of the RAS on the response of the mesentery to hypoxic alveolar macrophage supernatant.

Top: Representative microphotographs of the mesenteric microcirculation after topical application of 10 nM Ang II (A), hypoxic alveolar macrophage supernatant in rats pretreated with Ang II receptor blocker (B), and hypoxic alveolar macrophage supernatant in rats treated with NADPH oxidase inhibitor, apocynin, (C). The red arrows point to the mast cells; the blue arrows identify adherent leukocytes. Bottom: average values of leukocyte-endothelial adherence (LEA), leukocytes /100 μm, and intensity of mast cell degranulation (MCD) in arbitrary units. C and E: mean ± SEM of values at the end of the control and the experimental period, respectively. N=5 in all cases. * = P<0.01 vs. corresponding value in intact rats.

Figure 13 shows the effects of Ang II receptor blockade (Figure 13A, N=5) and of apocynin (Figure 13 B, N=5) on the response of the microcirculation to the basic mast cell secretagogue C48/80. When applied to the mesentery or cremaster microcirculation, C48/80 induces mast cell degranulation and leukocyte-endothelial adherence. In the present experiments, pretreatment with Ang II receptor blocker (Figure 13A) or with apocynin (Figure 13 B) did not

prevent the mast cell degranulation but inhibited the leukocyte-endothelial adherence induced by C48/80.

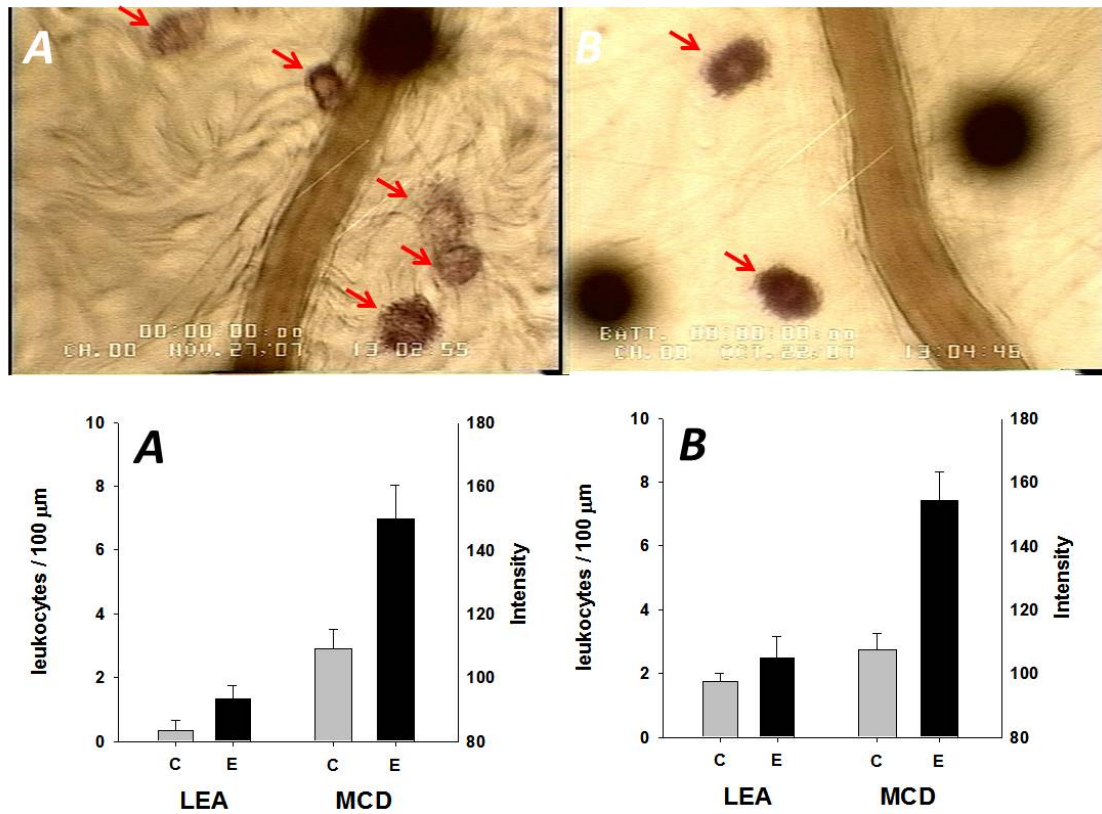


Figure 13 Role of the RAS on the microvascular response to C48/80

Top: Representative microphotographs of mesenteric microcirculation illustrating the effect of topical application of C48/80 after pretreatment with Ang II receptor blocker (A); or with apocynin (B). Bottom: average values of leukocyte-endothelial adherence (LEA), leukocytes /100 μ m, and intensity of mast cell degranulation (MCD) in arbitrary units. C and E: mean \pm SEM of values at the end of the control and the experimental period, respectively. N=5 in all cases. * = $P < 0.01$ vs. corresponding value in intact rats.

These results indicate that, as seen in the cremaster, the increased leukocyte-endothelial adherence produced in the mesentery by hypoxic alveolar macrophage supernatant is mediated by activation of the RAS; the RAS is

activated by mast cell degranulation induced, in turn, by an agent released into the supernatant by hypoxic alveolar macrophages. The effectiveness of the NADPH oxidase inhibitor apocynin on the inflammatory response further suggests that the effects of RAS activation are mediated by NADPH oxidase assembly.

5.3.2 Peritoneal mast cells contain active renin

The immunohistochemical results demonstrated that peritoneal mast cells express immunoreactive renin which was mainly contained in granules as shown in Figure 14B. No immunolabelling was detected in the control group incubated with secondary antibody only (Figure 14A). After treatment with mast cell secretagogue C48/80, the renin was detected in the granules released from mast cells (Figure 14 C).

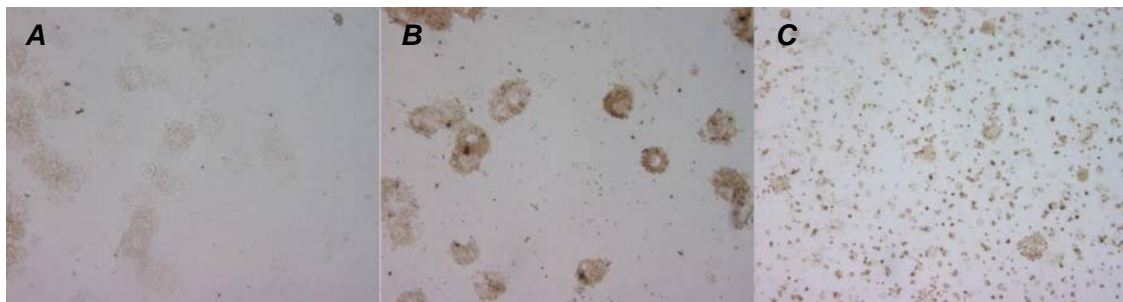


Figure 14 Presence of renin in rat peritoneal mast cells

Immunohistochemical staining with anti-renin antibody was detected in rat peritoneal mast cells (B). After activation with mast cell secretagogue C48/80, the renin was detected in the released granule (C).

The functional significance of mast cell renin was investigated in the following manner. Mast cells were cultured in plasma obtained from normal rats which would provide angiotensinogen, the substrate for renin. MCP-1 was added and formation of Ang II was measured by ELISA method. The results showed that MCP-1 induced Ang II production in a concentration-dependent manner, which was abolished by MCP-1/CCL2 receptor antagonist (Figure 15).

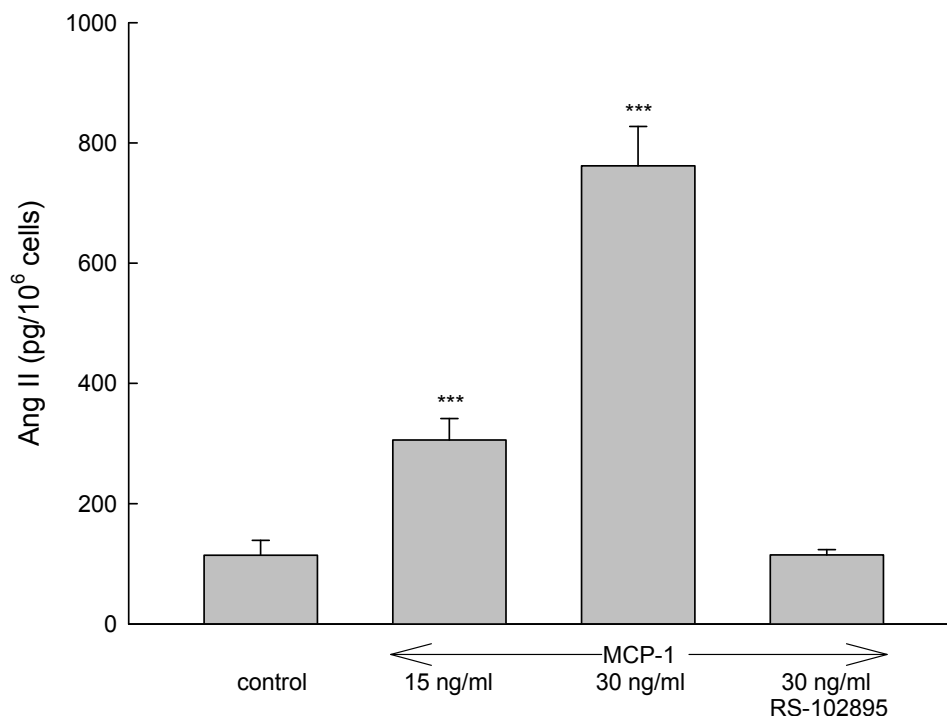


Figure 15 Effect of MCP-1 on the formation of Ang II

Peritoneal mast cells obtained from normoxic intact rats (0.4×10^6 cells) were immersed in 0.4 ml of plasma obtained from normal rat. The plasma contained RS-102895, MCP-1 or both. Data are mean \pm SE of 3 experiments per group. * = $P < 0.01$ vs. control.

Since degranulated mast cells were the only source of renin, the increased formation of Ang II was proportional to the elevated percentage of degranulated mast cells, which is confirmed by the evidence that application of renin inhibitor (WFML, 3 μ M) also blocked the formation of Ang II (Figure 16), suggesting the renin released from activated mast cells mediated Ang II production.

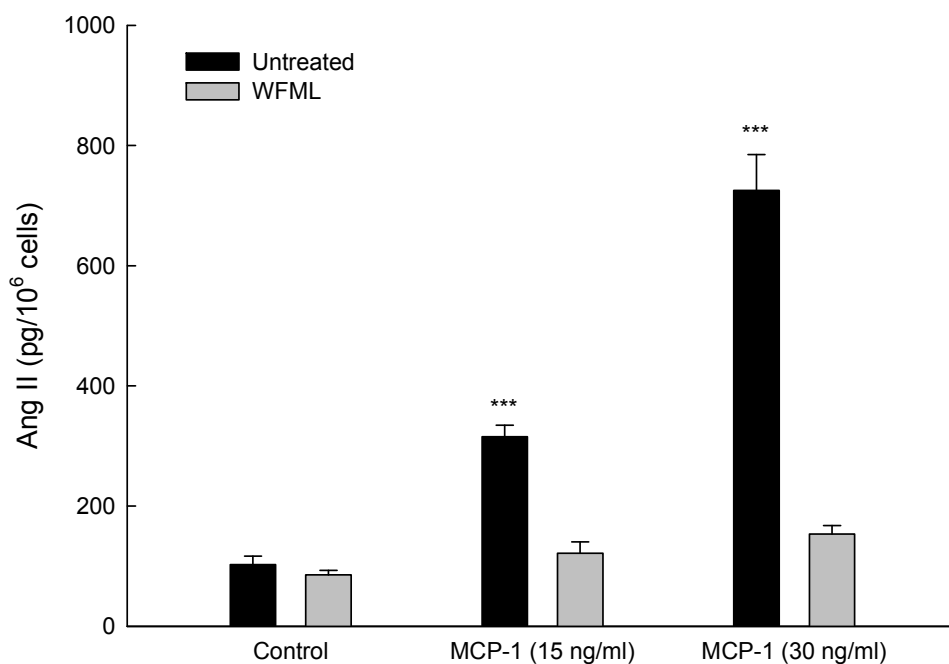


Figure 16 Effect of renin inhibitor on the formation of Ang II

Peritoneal mast cells obtained from normoxic intact rats (0.4×10^6 cells) were immersed in 0.4 ml of plasma obtained from normal rat. The plasma contained renin inhibitor (WFML), MCP-1 or both. Data are mean \pm SE of 3 experiments per group. * = $P < 0.01$ vs. corresponding control.

5.4 Discussion

The central findings of these experiments are the following: 1) topical application of Ang II or C48/80 behaved the same way in mesentery as that observed previously in the cremaster microcirculation; 2) Ang II receptor blocker abolished the inflammatory response induced by either C48/80 or hypoxic alveolar macrophage supernatant, which also occurred in cremaster. 3) Apocynin blocked the leukocyte-endothelial adherence, but not mast cell degranulation induced by either C48/80 or hypoxic alveolar macrophage supernatant. 4) Peritoneal mast cells contained renin which was released by degranulated mast cells and initiated the activation of local RAS. These findings demonstrate a direct link between mast cell degranulation and inflammation induced by activation of local RAS. The results add a key piece of evidence that confirms our hypothesis that the inflammation produced by alveolar hypoxia is triggered by alveolar macrophage-derived MCP-1 via RAS activation.

The RAS was blocked at two different sites: the interaction between Ang II and its receptors and NADPH assembly of NADPH. The Ang II receptor blocker we used blocks both type 1 and type 2 Ang II receptors. While most effects of Ang II are mediated by type 1 receptor stimulation, there is evidence (Piqueras et al., 2000) that activation of both receptor types is involved in the increased leukocyte-endothelial interactions promoted by exogenous Ang II. Thus, we selected an Ang II receptor blocker that would inhibit both type of receptor types.

Topical application of supernatant of alveolar macrophages equilibrated with 10% O₂ produced essentially the same response in the mesentery (Chao et al., 2009a) as that observed previously in the cremaster microcirculation (Gonzalez et al., 2007a): mast cell degranulation, increased leukocyte-endothelial adherence, and activation of the local RAS. As observed in the cremaster (Gonzalez et al., 2007a; Gonzalez et al., 2007b), activation of the RAS in the mesentery is a result of mast cell degranulation. Topical application of Ang II produced leukocyte-endothelial adherence, but not mast cell degranulation, which suggests that the effect of Ang II was downstream of mast cell activation. This was confirmed by the treatment of Ang II receptor blocker which abolished the leukocyte-endothelial adherence induced by hypoxic alveolar macrophage supernatant and C48/80, but not mast cell degranulation. Since alveolar hypoxia produced widespread inflammation in different tissues, the same observation in mesentery and cremaster support that the similar mechanisms are involved in this process.

In addition to demonstrate the similarity of responses in the cremaster and the mesentery, the present experiments expand our previous findings in the cremaster by demonstrating a participation of NADPH oxidase in this process: topical application of apocynin had essentially the same effects as the Ang II receptor blocker in preventing the inflammation induced by both hypoxic alveolar macrophage supernatant and C48/80. Previous studies have demonstrated that apocynin, an effective and selective inhibitor of NADPH oxidase, inhibited

peroxynitrite formation in murine macrophages (Muijsers et al., 2000) and vascular cell adhesion molecule 1 (VCAM-1) expression in endothelial cells (Matheny et al., 2000), increased glutathione synthesis, activated activator protein 1 (AP-1) in alveolar epithelial cells (Lapperre et al., 1999), and decreased the LPS-induced TNF- α expression in human monocytes (Stolk et al., 1994). All these properties of apocynin make it a promising anti-inflammatory agent *in vivo* (Zhou et al., 2002). Based upon this series of findings, it provides further insight into the mechanism of action of Ang II by showing that its inflammatory effects lie downstream of NADPH oxidase activation. A role for NADPH oxidase-generated ROS has been documented in several conditions characterized by increased RAS activity, including diabetes (Li and Shah, 2004), hypertension (Ferrario and Strawn, 2006; Hanna et al., 2002), and ischemia-reperfusion (Yusof et al., 2007), but the role of ROS in the inflammatory response induced by hypoxia remains unclear yet. The dependence of ROS levels on the PO₂ was observed in intact animals (Wood et al., 1999a), as well as in cardiomyocytes (Duranteau et al., 1998; Park et al., 1991) and hepatocytes (El-Bassiouni et al., 1998). The extent of leukocytes adherence during hypoxia was proportional to the increased in ROS level (Steiner et al., 2002). All of those studies suggest the participation of ROS in the inflammation induced by the hypoxia. The mechanism of ROS in this phenomenon needs further study.

While it is clear that the RAS is activated by mast cells (Gonzalez et al., 2007b), the mechanism by which mast cell degranulation activates the RAS was

not clear. The traditional RAS is defined by the formation of Ang I from angiotensinogen via kidney-derived renin, followed by conversion to Ang II via endothelial ACE (Davisson, 2003). Recently, the local generation of Ang II, which acts on resident receptor, has gained considerable attention. Renin is the enzyme that catalyzes the first and rate-limiting step of RAS. The release of renin has been demonstrated in myocardial mast cells (Mackins et al., 2006; Silver et al., 2004). Renin mRNA and protein has been detected in fibroblasts, as well as endothelial and coronary vascular smooth muscle cells and human mast cell lines (Bader et al., 2001; Dostal, 2000; Dostal and Baker, 1999; Endo-Mochizuki et al., 1995; Silver et al., 2004). Here, we provided evidence that renin is expressed in rat peritoneal mast cells, implying that renin from mast cells contributed to activation of tissue-specific RAS in inflammation induced by alveolar hypoxia. Furthermore, if MCP-1 is the mediator of inflammation induced by alveolar hypoxia, it would be expected that MCP-1 activates the release of renin from degranulated mast cells. This appears to be the case: MCP-1 induced Ang II production in a concentration-dependent manner, which was blocked by pretreatment of MCP-1/CCL2 receptor antagonist. Additional experiments were conducted in the presence of 3 μ M WFML, a specific rat renin inhibitor, to verify that the Ang II-generating activity in the supernatant was indeed renin. The results suggest that the formation of Ang II was due to renin because the renin inhibitor abolished the increased of Ang II induced by MCP-1.

All data in the present experiments suggest that MCP-1 stimulate release of renin from mast cells, which, in turn, activates local RAS. However, the signaling pathways through which MCP-1 triggers release of renin from mast cells are still poorly documented. The MCP-1 triggers several signal transduction pathway, including extracellular signal-related kinases (ERKs) and PI3-kinase (PI3-K) pathway (Cambien et al., 2001). The pathway involved in our case need further studies.

In summary, the present experiments provide key evidence in support of our hypothesis that the inflammation of alveolar hypoxia is initiated by the release of MCP-1 from alveolar macrophages. MCP-1, in turn, induced Ang II production which was inhibited by CCL2 receptor antagonist and rennin inhibitor, indicating that local mast cell-derived rennin was involved in Ang II production in this process.

CHAPTER 6

Summary of findings and future directions

6.1 Summary of major findings

The central findings of this project are the following: 1) There is a direct link between alveolar macrophage activation by hypoxia and mast cell degranulation. The results also rule out the possible role of low tissue PO_2 on the resident tissue macrophages or tissue mast cells in the early phase of the inflammation of alveolar hypoxia. 2) MCP-1 plays a central role in the initiation of the systemic inflammatory response to alveolar hypoxia. A co-factor, normally present in normoxic plasma and originated in alveolar macrophages, is necessary for the stimulatory effect of MCP-1 on mast cells. 3) Mast cell-derived renin initiates the activation of local RAS which mediates the inflammation induced by alveolar hypoxia.

First, supernatant of hypoxic alveolar macrophages elicited an inflammatory response in the normoxic mesentery, while supernatant of peritoneal macrophages equilibrated with even lower PO_2 values had no inflammatory effect. On the other hand, alveolar macrophages, but not peritoneal macrophages responded with a transitory release of H_2O_2 when exposed to hypoxia. The H_2O_2 release is a manifestation of the respiratory burst which occurs during macrophage activation and is characterized by superoxide generation followed by dismutation to H_2O_2 . Mast cell degranulation did not occur

when mast cells were directly exposed to hypoxia for a period consistent with the time course of the initiation of the inflammation; however degranulation was observed when the mast cells were immersed in supernatant of alveolar macrophages that had been equilibrated in hypoxia. Thus, both *in vivo* and *in vitro* findings demonstrate a link between activation of alveolar macrophages by hypoxia and mast cell degranulation. These results also show that neither reduced local PO₂ nor activation of resident tissue macrophages participate in the initiation of the systemic inflammation of hypoxia.

Second, primary cultures of alveolar macrophages, but not of peritoneal mast cells or peritoneal macrophages, released MCP-1 within 30 minutes of exposure to hypoxia. Plasma MCP-1 levels in conscious rats breathing 10 % O₂ increased during the first 30 minutes of hypoxia; this increase was abolished by depletion of alveolar macrophages. Immersion of mast cells in the plasma drawn from the intact conscious rats breathing 10% O₂, in which an elevated MCP-1 level was documented, produced substantial degranulation, while degranulation did not occur when mast cells were immersed in plasma of alveolar macrophage-depleted rats, which had lower MCP-1 concentration. A role of MCP-1 in the systemic inflammation of hypoxia in the intact animal was further supported by the observation that pretreatment of the mesentery with RS-102895, a selective antagonist for CCR2b (the putative MCP-1/CCL2 receptor expressed in mast cells), prevented the rapid degranulation of perivascular mast cells and the

increased leukocyte-endothelial adherence to mesenteric venules which was observed in intact rats breathing 10% O₂.

Addition of MCP-1 to normoxic plasma or normoxic alveolar macrophages supernatant produced concentration-dependent degranulation of immersed mast cells. The percent of degranulated mast cells immersed in the plasma samples drawn from the intact rats breathing 10% O₂ was close to that seen when mast cells were immersed in normoxic plasma or normoxic alveolar macrophages supernatant with similar MCP-1 concentrations (Figure 8). These results indicate that the plasma MCP-1 level reached by the hypoxic rats with normal alveolar macrophages count is sufficient to induce mast cell degranulation, suggest that mast cell degranulation occurred as a result of the elevated MCP-1 concentration, and was not the result of other factors associated with hypoxia.

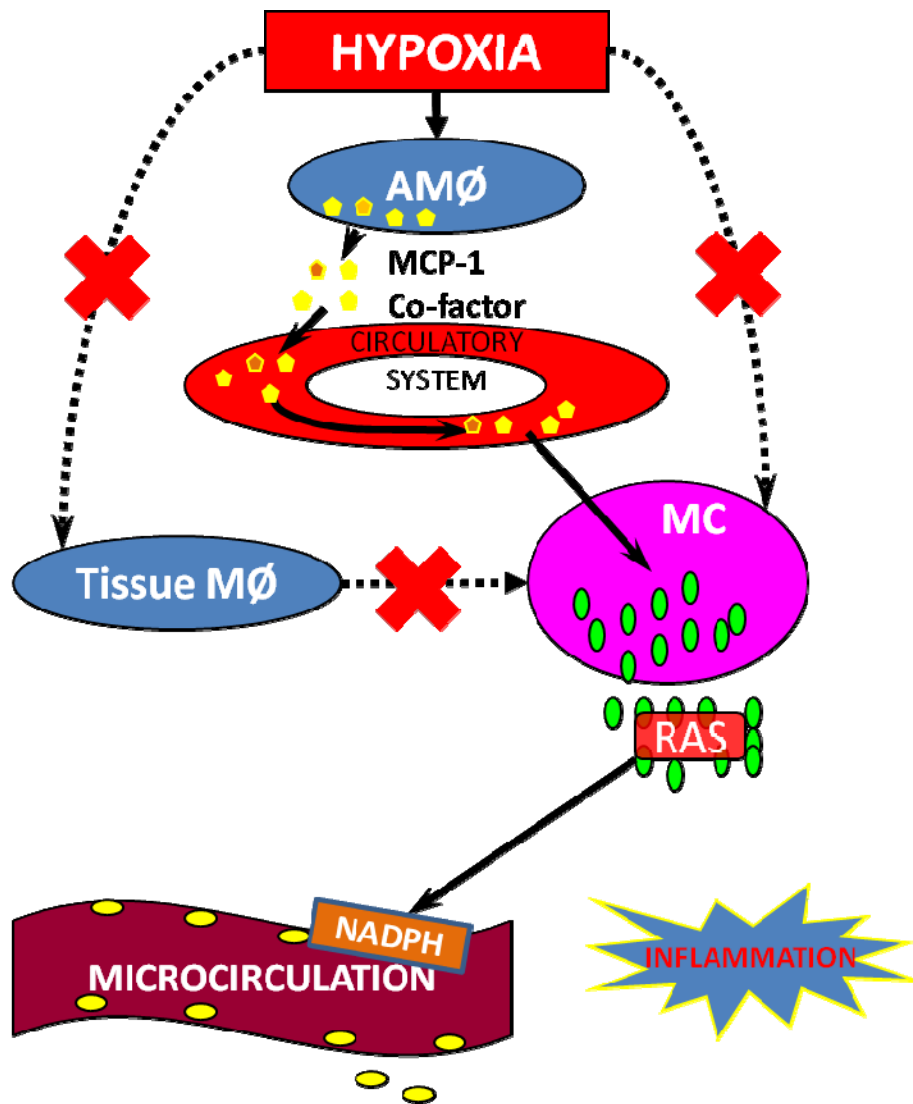
The possibility of a co-factor was initially suggested by the observation that MCP-1 did not produce degranulation of mast cells immersed in normoxic cell culture medium, even at concentrations that were effective in plasma or in alveolar macrophage supernatant. The results of the filtration experiments suggest that the co-factor was composed of at least two fractions: one with a molecular weight between 30 and 50 kDa and the other with a molecular weight between 50 and 100 kDa. The co-factor is constitutively secreted by alveolar macrophages and is normally present in plasma and extracellular fluid, which is suggested by several lines of evidence: 1) mast cells immersed in plasma obtained from alveolar macrophage-depleted rats after 60 min of 10 % O₂

breathing did not undergo degranulation. This was in spite of the fact that this sample exhibited a MCP-1 concentration that produces degranulation of 40-50 % of mast cells immersed in plasma from intact rats or in alveolar macrophages supernatant; 2) topical MCP-1 dissolved in serum-free DMEM replicated the response to hypoxia in the mesentery of intact, normoxic rats, while it had no effect in the alveolar macrophage-depleted rats; 3) MCP-1 dissolved in plasma of rats with normal alveolar macrophages count did produce an inflammatory response in the alveolar macrophages –depleted rats. Although these results provide persuasive evidence of an alveolar macrophage-borne co-factor of MCP-1, further study will be needed to dissect its exact nature

Third, Ang II induced leukocyte-endothelial adherence without producing mast cell degranulation, which suggest that activation of RAS occurs downstream of mast cell degranulation. Ang II receptor blocker inhibited the leukocyte-endothelial adherence, but not mast cell degranulation induced by either hypoxic alveolar macrophage supernatant or C48/80, which suggest that inflammatory response is mediated by RAS. The source of rennin was peritoneal mast cell as confirmed by immunocytochemistry. Renin released from the degranulated mast cell in turn activated local RAS, which initiated a cascade of events resulting in the local inflammatory response.

In summary, the present studies provide substantial evidence in support of the idea that the systemic inflammatory response to alveolar hypoxia is initiated by an alveolar macrophage-borne mediator, which, in turn, activate mast cells and

local RAS. The results also provide compelling evidence that this mediator is MCP-1. The sequence of events presents schematically in the following figure:



6.2 Future directions

The key finding of the current experiments is that MCP-1 released from alveolar macrophages initiates the cascade of inflammation. The time course of release of MCP-1 suggests that this is not due to changes in gene expression of MCP-1. It is likely that MCP-1 stored in alveolar macrophages is released in response to the stimulation of hypoxia. The mechanism by which hypoxia triggers the release of MCP-1 from alveolar macrophage is not clear. On the other hand, hypoxia may induce release of MCP-1 from other type cells, which is suggested by the observation that the level of MCP-1 increased in plasma from alveolar macrophage-depleted rats exposed to 10% O₂ for 60 minutes. The stimulus, as well as the role of the delayed release of MCP-1, is not known. Interestingly, this plasma did not induced mast cell degranulation *in vivo*, while the MCP-1 dissolved in normoxic plasma or alveolar macrophage supernatant in the same concentration induced mast cell degranulation. These results suggest the existence of co-factor in normoxic plasma or alveolar macrophage supernatant for effect of MCP-1 on mast cells. Further experiments will be undertaken to identify the nature of the candidate co-factor.

MCP-1 activated mast cells either *in vivo* or *in vitro* system, which is mediated primarily through its cognate receptor, CCR2, expressed in mast cells. Pathways triggered by MCP-1 include the following: induction of a pertussis toxin (PTX)-sensitive rise of intracellular calcium, inhibition of adenyl cyclase, phospholipase C activation, activation of extracellular signal-related kinases (ERKs), stimulation

of 2 separate PI 3-kinase isoforms, namely p85/p110 PI3-kinase (PI3-K) and PI3K-C2 α . However, which signal transduction pathway is involved in mast cell degranulation induced by alveolar macrophage-derived MCP-1 remains unknown and need further investigation.

Previous and current studies in cremaster and mesenteric microcirculation suggest the participation of RAS in inflammation induced by alveolar hypoxia. There are two possible mechanisms by which mast cells may activate the RAS: chymase- Ang II forming pathway and ACE- Ang II forming pathway. Both ACE inhibition and Ang II receptor blockade attenuate the inflammatory effects in cremaster microcirculation induced by either hypoxia or mast cell secretagogue C48/80, which suggest that the ACE-Ang II forming pathway plays a role in the cremaster microcirculation bed. Whether the ACE-Ang II forming pathway plays a same role in other types of microcirculation bed, such as mesenteric microcirculation, remains poorly defined. We have shown that renin inhibitor blocked the formation of Ang II induced by MCP-1 in cultured mast cells. The experiments carried out in mast cells with ACE inhibitor will help to answer this question.

Moreover, previous and current studies suggest that mast cell degranulation is a key event in the inflammation induced by alveolar hypoxia. Mast cells are abundant in the microcirculatory beds in which the inflammatory effects of hypoxia have been demonstrated. Mast cells are known to degranulate and release various inflammatory mediators that are either preformed, such as

histamine, proteoglycans, and tryptase, or synthesized, including leukotriene B₄, platelet activating factor, prostaglandins, and cytokines, some of which have been shown to participate in the systemic inflammation of hypoxia, such as leukotriene B₄ and platelet activating factor. Whether other products released from degranulated mast cells are involved in the inflammation induced by hypoxia will be subject to further study.

In summary, the present experiments provide strong evidence to support our hypothesis that the mediator released from alveolar macrophage initiates the cascade of inflammation by activation of mast cells and local RAS. The proposed experiments will lend strength to understand the detail mechanism of this phenomenon.

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